UNESP - Universidade Estadual Paulista

"Júlio de Mesquita Filho"

Faculdade de Odontologia de Araraquara

SÂMIA CRUZ TFAILE CORBI

Avaliação da Expressão Gênica e de Lesões no DNA de Indivíduos Portadores de Diabetes Mellitus Tipo 2, Dislipidemia e Periodontite Crônica

Araraquara

2014

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Tese apresentada ao Programa de Pós-Graduação em Odontologia – Área de Periodontia, da Faculdade de Odontologia de Araraquara, da Universidade Estadual Paulista para título de Doutor em Odontologia.

Orientadora:
Profa. Dra. Raquel Mantuaneli Scarel Caminaga

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Avaliação da Expressão Gênica e de Lesões no DNA de Indivíduos Portadores de Diabetes Mellitus Tipo 2, Dislipidemia e Periodontite Crônica

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Dedico este trabalho....

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Corbi SCT. Avaliação da expressão gênica e de lesões no DNA de indivíduos portadores de diabetes mellitus tipo 2, dislipidemia e periodontite crônica [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2014.

Resumo

O objetivo deste estudo foi avaliar a expressão gênica de indivíduos portadores de diabetes mellitus tipo 2 (DM2, compensados e não compensados metabolicamente), dislipidemia e/ou periodontite crônica, e avaliar se tais alterações metabólicas apresentam efeito mutagênico. Cento e cinquenta pacientes, divididos em 5 grupos (grupo 1 - diabetes descompensado, com dislipidemia e com doença periodontal; grupo 2 - diabetes compensado, com dislipidemia e com doença periodontal; grupo 3 - sem diabetes, com dislipidemia e com doença periodontal; grupo 4 - sem diabetes, sem dislipidemia e com doença periodontal; e o grupo 5 - sem diabetes, sem dislipidemia e sem doença periodontal), foram avaliados quanto ao exame periodontal completo, exame físico e avaliação laboratorial da glicemia de jejum e perfil lipídico. De cada paciente foi coletado sangue para investigar a expressão gênica e as lesões no DNA. A avaliação da expressão gênica foi realizada por *microarray* e validada por RT-qPCR (Transcrição Reversa seguida de Reação em Cadeia da Polimerase em Tempo Real, ou quantitativo). As lesões no DNA foram avaliadas por meio do teste do micronúcleo. Os dados foram submetidos à análise bioinformática e estatística. Para verificar os resultados obtidos pelo *microarray*, os Grupos 1, 2 e 3 foram submetidos a comparações por pares. As análises de RT-qPCR confirmaram a expressão diferencial dos genes *HLA-QA1, PDCD6, TRDV3, PPAP2B, HLA-DQB1, RIN3, VCAN, PPIC* e *SLC6A13*. As frequências de micronúcleos foram significativamente mais elevadas em pacientes afetados por pelo menos uma das doenças sistêmicas, em comparação com aqueles

sistemicamente saudáveis. Concluímos que foram identificados genes diferencialmente expressos em indivíduos portadores de DM2 também afetados por dislipidemia e periodontite crônica. Os resultados do micronúcleo confirmaram ser este teste útil como biomarcador para lesões no DNA, e demonstraram que as três patologias, ocorrendo simultaneamente, promoveram um papel adicional na produção de danos no DNA.

Palavras-chave: Expressão gênica, Doenças periodontais, Diabetes Mellitus tipo 2, Testes para micronúcleos.

Corbi SCT. Gene expression and DNA damage in individuals with type 2 diabetes mellitus, dyslipidemia and chronic periodontitis [Tese de Doutorado]. Araraquara: Faculdade de Odontologia da UNESP; 2014.

Abstract

The aim of this study was to evaluate the gene expression in individuals with type 2 diabetes (T2D, poor and well-controlled diabetics), dyslipidemia, and/or chronic periodontitis, and to assess whether these metabolic changes have mutagenic effect. One hundred and fifty patients were divided into 5 groups (group 1 – poor controlled diabetes with dyslipidemia and periodontal disease; group 2 – well-controlled diabetes with dyslipidemia and periodontal disease; group 3 – without diabetes with dyslipidemia and periodontal disease; group 4 – without diabetes, without dyslipidemia and with periodontal disease; and group 5 – without diabetes, dyslipidemia and periodontal disease), and were assessed a complete periodontal and physical examination, and laboratory evaluation of fasting glucose and lipid profile. From each patient, blood was collected to investigate gene expression and DNA damage. The gene expression was evaluated by *microarray* analysis and validated by RTqPCR (Polymerase Chain Reaction Real-Time, or quantitative). The DNA damage was evaluated using the micronucleus test. Data were subjected to statistical and bioinformatics analyses. To verify the results obtained by microarray, the Groups 1, 2 and 3 were submitted to pair comparisons. The RT-qPCR analysis confirmed the differential expression of *HLA-QA1, PDCD6, TRDV3, PPAP2B, HLA-DQB1, RIN3, VCAN, PPIC* and *SLC6A13* genes. Significantly higher micronuclei frequencies were found in patients affected by any of the systemic diseases in comparison with the ones systemically healthy. We concluded that differentially expressed genes were identified

in individuals with type 2 diabetes, dyslipidemia and chronic periodontitis. The results of the micronucleus confirmed that this test is useful as biomarker for DNA damage, and demonstrated that the three pathologies occurring simultaneously promoted an additional role in the production of DNA damage.

Keywords: Gene expression, Periodontal diseases, Type 2 diabetes mellitus, micronuclei.

Lista de Abreviaturas e Nomenclaturas

ABL1 – Gene *Abelson murine leukemia viral oncogene homolog 1*

ACSL3 – Gene *Acyl-coa synthetase long chain family, member 3*

ACTG1 – Gene *Actin, gamma-1*

ADC- Adenocarcinoma

ADCY7 – Adenylate cyclase type 7 enzyme

AGBL5 – Gene *ATP/GTP Binding Protein-Like 5*

AGE - Produtos finais da glicação avançada (Advanced Glycation End-products)

AIP1 - Actin interacting protein 1

AKT2 – RAC-beta serine/threonine-protein kinase enzyme

BIRC-1 – Proteína inibidora de apoptose - 1

BMI – Body mass Index

BOP – Bleeding on Probing

bp – Base pair (par de bases)

BRCA2 – Gene *Breast cancer 2, early onset*

CAL – Clinical attachment Level

CBMN - Cytokinesis-block micronucleus

CCDN3 – Gene *Cyclin D3*

CCR4 – C-C chemokine receptor type 4 protein

CCL28 – Gene *Chemokine (C-C Motif) Ligand 28*

cDNA - DNA complementar

cRNA – RNA complementar

C3 – Compinente do Complemento 3

CD4+ - Linfócito T4

CD8+ - Linfócito T citotóxico

CD14 – Monocyte differentiation antigen CD14

CD38 – Cyclic ADP ribose hydrolase

CD74 – HLA class II histocompatibility antigen gamma chain / HLA-DR antigens-associated invariant chain

CEBPD – Gene *CCAAT/Enhancer Binding Protein (C/EBP), Delta*

CEP – Comitê de Ética em Pesquisa

C-FOS – Finkil-Biskis-Junkins oncogênese do vírus murinho osteossarcoma

cm - centímetro

CSPG2 – Chondroitin sulfate proteoglycan 2

CT - Threshold cycle

CTCs – Circulating tumor cells

°**C** – Grau Celsius

CyCAP – Cyclophilin-C-associated protein

COX2 – Prostaglandina- endoperóxido sintase - 2

CXCL-3 – Proteína Inflamatória do macrófago – 2B

DAPK1 – Death-associated protein kinase

DM - Diabetes Mellitus

DM1 - Diabetes Mellitus tipo 1

DM2 - Diabetes Mellitus tipo 2

DNA - Ácido Desoxirribonucleico

DP - Desvio Padrão

DSC-1 – Desmocolina-1

DSCR-1 – Proteína da região crítica da Síndrome de Down-1

ECM – Matriz extracelular

EDN-1 – Endotelina-1

ENO2 – Gene *Enolase 2*

FAS - Fas ligand (FasL or CD95L)

FII-15 – Fator indutor de interferon-15

FC – Fold change

FCRL5 – Gene *Fc receptor-like protein 5*

GABA – Gamma-aminobutyric acid

GAPDH – Gene *Glyceraldehyde 3-phosphate dehydrogenase*

GAT2 – Gaba transporter 2

GBI – Gingival bleeding index

GNL1 – Guanine Nucleotide Binding Protein-Like 1

GZMM – Granzyme M protein

G3BP1 – Nucleic-acid-binding protein with a proposed helicase activity

HbA1c – Glycated Hemoglobin A1c fraction

HDL – High density lipoprotein

HLA – Human leukocyte antigenes

HLA-DQA1 - Gene *Major Histocompatibility Complex, Class II, DQ-Alpha-1*

HLA-DQB1 – Gene *Major Histocompatibility Complex, Class II, DQ-Beta-1*

HMG – High-Mobility group

HPGDS – Gene *Hematopoietic Prostaglandin D Synthase*

HSPA4 – Gene *Heat Shock 70kDa Protein 4*

hs CRP – High Sensitive C-reactive Protein

ICAM - Gene *Molécula de Adesão Intercelular*

IFN- γ – Interferon-gama

Ig - Imunoglobulina

IGF2 – Fator de crescimento de Insulina 2

IGHA1 – Gene *Immunoglobulin Heavy Constant Alpha 1*

IL- Interleucina

IL12RB2 – Gene *Interleukin 12 Receptor, Beta 2*

IMC- Índice de massa corporal

iNOS – Inducible nitric oxide synthase

INSR – Gene *Insulin Receptor*

IPV- Índice de placa visível

ISM- Índice de sangramento marginal

IRF-7 – Fator de regulação do Interferon-7

ITP – Púrpura trombocitopênica

JNK1 – c-Jun N-terminal kinases

Kb – Kilobase

KDa – Kilodalton

Kg – Kilo

KIT – Receptor tyrosine kinase

KRT2A – Queratina 2A

LDL – Low Density Lipoprotein

LDL-AGE – LDL ligado à produtos finais da glicação avançada

LDL-oxidized – LDL-oxidada

LGALS12 – Gene *Lectin, Galactoside-Binding, Soluble, 12*

LIFR – Gene *Leukemia Inhibitory Factor Receptor Alpha*

LPA – Lysophosphatidic acid

LPP3 – Lipid Phosphate Phosphatase 3

LPS – Lipopolissacarídeos

LTF – Lactotransferrina

m – Metro

MAP2K5 – Gene *Mitogen-Activated Protein Kinase Kinase 5*

MAP3K5 – Gene *Mitogen-Activated Protein Kinase Kinase Kinase 5*

MCM4 – Gene *Minichromosome Maintenance Complex Component 4*

MCP-1 – Proteína quimiotática de monócitos - 1

MDA – Malondialdehyde

MDM2 – Gene *MDM2 Oncogene, E3 Ubiquitin Protein Ligase*

MGC5566 – Proteína hipotética de Homo Sapiens

MHC – Major histocompatibility Complex

miRNA – Micro RNA

mm – Milímetros

mg/dL – Miligramas por decilitro

mL - Mililitros

MMP – Metaloproteinase

MN - Micronúcleo

MNF – Micronuclei frequency

MCF – Frequência de células binucleadas com micronúcleo

mRNA – RNA mensageiro

MYL6B – Gene *Myosin, Light Chain 6B, Alkali, Smooth Muscle And Non-Muscle*

NDI – Índice de Divisão Nuclear

NF-kB – Nuclear factor kappa B

NI- Nível de inserção

ng - Nanôgrama

nm – Nanômetro

NOS2 – Nitric oxide synthase 2

NOX1 – Gene *NADPH Oxidase 1*

NSCLS – Non-small-cell lung carcinoma

OMIM – Online Mendelian Inheritance in Man

OPG – Osteoprotegerina

Ox-LDL – Oxidized LDL

PAI - Inibidor do ativador de plasminogênio tecidual

PBMC – Células mononucleares do sangue periférico

PBRM1 – Gene *Polybromo 1*

PCR – Reação em Cadeia da Polimerase (Polymerase Chain Reacion)

PDCD6 – Gene Programmed Cell Death 6

PDAP1 - PDGFA Associated Protein 1

PD – Probing Depth

PDE3A – Gene *Phosphodiesterase 3A, CGMP-Inhibited*

PECAM1 – Gene *Platelet/Endothelial Cell Adhesion Molecule 1*

PKM2 – Quinase Piruvato tipo M2

PLD1 – Gene *Phospholipase D1, Phosphatidylcholine-Specific*

PMN – Células Polimorfonucleares

PPAP2B – Gene *Phosphatidic acid phosphatase type 2B*

PPAPDC2 – Gene *Phosphatidic Acid Phosphatase Type 2 Domain Containing 2*

PPP2CB – Gene *Protein Phosphatase 2, Catalytic Subunit, Beta Isozyme*

PPIC – Gene *Peptidyl-Prolyl Isomerase C*

PRF1 – Gene *Perforin 1 (Pore Forming Protein)*

PS- Profundidade de Sondagem

PTPNI – Proteína tirosina fosfatase de tipo não-receptor 1

qPCR - PCR Quantitativo em Tempo Real

RAB5 – Proteínas rab5 de Ligação ao GTP

RAGE- Receptor of advanced glycation end-products

RANKL- Receptor activator of nuclear factor kappa B

RAS - ***RA****t Sarcoma vírus*, ou vírus do sarcoma de rato; proteína

RCC2 - Regulator of Chromosome Condensation 2 protein

RHOBTB2 – Gene *Rho-Related BTB Domain Containing 2*

RIN3 – Gene *Ras and Rab interactor 3*

RIP-2 – Receptor-interagindo serina/terosina quinase-2

RGS-1 – Sinalizador da proteína G reguladora-1

RNA - Ácido Ribonucleico

RPL7A – Gene *Ribosomal Protein L7a*

RPTOR – Gene *Regulatory Associated Protein of MTOR, Complex 1*

RSS – Reconbination signal sequences

RT – Reverse Transcription

RT-qPCR – Transcrição Reversa seguida de Reação em Cadeia da Polimerase em Tempo Real, ou quantitativo

RORC – Receptor único C relacionado a RAR

ROS – Espécies reativas do oxigênio (Reactive Oxygen Species)

SALL3 – Gene *Sal-Like 3 (Drosophila)*

SCF – Stem cell factor

SD – Standard deviation

SEC13 – Gene *SEC13 Homolog (S. Cerevisiae)*

SH3 – SRC Homology 3 Domain protein domain

SH3BP4 – Gene *SH3-Domain Binding Protein 4*

SLC6A13 – Gene *Solute carrier family 6 – neurotransmitter transporter, gaba – member 13*

SOS1 – Gene *Son of Sevenless Homolog 1 (Drosophila)*

SS – Sangramento à sondagem

TCLE- Termo de consentimento livre e esclarecido

TCR – T-cell receptor

T2D – Type 2 Diabetes

TG- Triglicérides

TGF-B1 - Transforming growth factor, beta 1

TIMP – Inibidor de Metaloproteinase

TLRs – Toll-like receptors

TMSB4X - Thymosin beta-4 protein

TNF-α - Fator de necrose tumoral alpha

TRDV3 – Gene *T-cell receptor delta chain variable gene cluster*

µl – Microlitro

U/L – Unidades por Litro

VAMP2 – Gene *Vesicle-Associated Membrane Protein 2 (Synaptobrevin 2)*

VCAN – Gene *Versican*

VPI – Visible Plaque Index

XPA – Gene *Xeroderma Pigmentosum, Complementation Group A*

ZNF479 – Gene *Zinc Finger Protein 479*

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1 INTRODUÇÃO

O Diabetes Mellitus é uma doença metabólica que envolve primariamente os carboidratos, seguido dos lipídeos e proteínas, sendo caracterizado pela hiperglicemia resultante de defeitos na secreção de insulina, em sua ação ou em ambos. Como resultado direto da hiperglicemia e do desequilíbrio osmótico, uma tríade clínica clássica de sintomas é desenvolvida, e inclui polifagia, polidipsia e poliúria.

O aparecimento da doença está relacionado a fatores de risco genéticos, ambientais e comportamentais [55]. A prevalência do diabetes mellitus aumenta em todo o mundo, sendo estimado que mais de 300 milhões de indivíduos sejam afetados até 2030 [118]. Atualmente, os tipos mais comuns de DM são tipo 1 (DM1), em que as células beta do pâncreas são destruídas por autoimunidade, levando à uma deficiência absoluta de insulina, e o tipo 2 (DM2), onde há graus variados de diminuição de secreção e resistência à insulina [2]. Muitos pacientes portadores de diabetes, particularmente os que manifestam o tipo 2 da doença, permanecem sem diagnóstico por muitos anos, uma vez que a hiperglicemia aparece gradualmente, gerando pouca sintomatologia no início da doença [21].

Um fator associado às complicações do diabetes é o processo não enzimático de glicação de proteínas, lipídeos e ácidos nucléicos, com subseqüente formação de produtos finais da glicação avançada (AGEs, *Advanced Glycation End-products*) [13]. Tanto macrófagos como células endoteliais apresentam receptores específicos para AGEs denominados RAGEs e esta interação resulta em diversas alterações celulares como o aumento da permeabilidade vascular e estímulo à secreção de citocinas pró-inflamatórias, como interleucina 1ß (IL-1ß) e fator de necrose tumoral alfa (TNF-α) [60, 120].

Além das alterações relacionadas à hiperglicemia citadas anteriormente, estas podem vir acompanhadas ainda da dislipidemia, a qual inclui mudanças qualitativas e quantitativas das lipoproteínas e transtornos no metabolismo dos lipídeos [46, 76]. Efeitos do microambiente dislipidêmico podem ser alterados no diabetes devido à presença de AGEs, os quais podem agir como co-estimuladores das células. A hiperglicemia associada ao diabetes não compensado aumenta a formação de AGEs, incluindo LDL-AGE, bem como está associada à maior quantidade de LDL-oxidada. Tanto AGEs como LDL-oxidada, presentes concomitantemente no sangue de pacientes com diabetes apresentam diversos efeitos biológicos sobre linfócitos e monócitos, os quais podem participar de diversas patologias associadas ao diabetes, como as doenças vasculares, menor resistência a infecções e maior severidade da doença periodontal [122].

A periodontite pode ser reconhecida como a sexta maior complicação associada ao diabetes [64], sendo que foi detectada maior extensão e severidade da doença periodontal em indivíduos portadores de diabetes do que naqueles não portadores da doença [65, 72, 84]. Entretanto, segundo Kinane, Bartold [54] (2007) o diabetes pode ser considerado mais um fator modificador da periodontite já existente do que propriamente um agente causador da doença.

Quanto maior a duração do diabetes, maior a prevalência e severidade de suas complicações [72, 107] e alguns estudos definem o controle metabólico como um fator de influência para a maior severidade da doença periodontal, com maior comprometimento para indivíduos pobremente controlados metabolicamente [61, 109], enquanto outros não evidenciaram tal relação [12, 103]. Alterações na resposta imunoinflamatória também representam um importante fator para o aumento da prevalência e severidade da destruição periodontal nos pacientes com diabetes [72], sendo que nos neutrófilos tais alterações podem ser verificadas quanto à aderência, quimiotaxia e fagocitose. Já

monócitos e macrófagos apresentam resposta exacerbada, o que resulta na produção de mediadores e citocinas pró-inflamatórias em níveis acima daqueles verificados em pacientes sem diabetes, mesmo em sítios com periodontite leve [97]. O nível de citocinas inflamatórias no fluido gengival parece estar correlacionado com o controle metabólico, tendo sido verificado que indivíduos descompensados metabolicamente podem apresentar níveis de IL-1β cerca de duas vezes maior quando comparados a indivíduos compensados [22].

As espécies reativas de oxigênio (ROS) são produzidas continuamente sob condições fisiológicas pelas células, sendo consideradas bioprodutos normais do metabolismo [83]. Durante a fagocitose esses radicais livres são liberados em maior quantidade como parte da reação bactericida [104]. As ROS são instáveis e apresentam um ou mais elétrons desemparelhados, o que confere a eles certo grau de reatividade [110]. Apesar de desempenharem funções fisiológicas, os radicais livres têm sido associados à patogênese de diversas desordens [95], entre estas o diabetes mellitus tipo 2 (DM2), dislipidemia e periodontite crônica. Sabe-se que ocorre uma produção de espécies reativas de oxigênio acima do normal em sítios de inflamação crônica [39] e que tais compostos deixam de exercer uma função meramente fisiológica, passando a contribuir para a injúria inflamatória dos tecidos do hospedeiro [104]. As ROS podem causar danos oxidativos a um grande número de moléculas como carboidratos, lipídios e o DNA. Em condições de maior produção de radicais livres, as células podem não responder adequadamente aos mecanismos antioxidantes. Apesar de haver outros processos envolvidos, o excesso de glicose é o principal responsável pela grande produção de radicais livres em pacientes diabéticos. Além disso, pacientes com DM2 apresentam uma defesa antioxidante deficiente em relação a indivíduos normais [10, 78].

O aumento na produção de ROS também tem sido atribuído a glicação de proteínas [7, 36] e/ou a auto-oxidação da glicose durante o processo de hiperglicemia [48]. Sendo assim, além dos AGEs, o estresse oxidativo passa a apresentar um papel importante tanto na patogênese como nas complicações do diabetes [52]. Adicionalmente, a hiperglicemia pode acarretar a glicação de enzimas antioxidantes, causando sua inativação [117].

A expressão gênica tem sido investigada isoladamente nas patologias DM2, dislipidemia e doença periodontal. Nozaki et al. [80] (1997) examinaram a expressão de várias citocinas e enzimas utilizando biópsia de tecido gengival pela técnica de RT-PCR (transcrição reversa/ reação em cadeia da polimerase). Os autores obtiveram tecido gengival clinicamente saudável e/ou inflamado de pacientes com periodontite crônica e periodontite agressiva. Foi observado que os níveis de mRNA de IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-15, TNF-α e interferon gama (IFN-γ) foram maiores em tecido gengival inflamado do que em tecido não inflamado de pacientes com periodontite crônica. Também foi detectada a expressão de TNF-α e IL-12 em tecido periodontal inflamado [93], e maior expressão de MCP-1, seu receptor CCR4 e maior expressão de IL-10 [31]. Em comparação com indivíduos periodontalmente saudáveis, indivíduos com doença periodontal também expressaram maiores níveis de metaloproteinases (MMPs 1, 2 e 9), inibidores de metaloproteinases (TIMPs 1, 2 e 3), OPG (osteoprotegerina), RANKL, TNF-α, IFN-γ, IL-4 e IL-10.

Wang et al. [113] (2003) examinaram a expressão de IL-1α, IL-1β, IL-6, IL-8, TNF-α, CD14, TLR2 e TLR4 em fibroblastos gengivais humanos obtidos de gengiva saudável ou inflamada usando *microarray*. Os níveis de expressão dos oito genes foram significativamente maiores no grupo com gengiva inflamada do que no grupo com gengiva saudável, indicando que tais genes estão envolvidos no processo inflamatório

da doença periodontal. Kim et al. [53] (2006) avaliaram a hipótese de que pacientes com periodontite refratária apresentariam múltiplos genes hiper e/ou hipoexpressos que poderiam influenciar o risco clínico dessa doença. Utilizando a técnica de *microarray* identificaram 68 genes hiperexpressos e 6 genes hipoexpressos. Foram validados por qPCR cinco genes hiperexpressos (lactotransferrina [LTF], metaloproteinase de matriz 1 [MMP-1], [MMP-3], fator indutor de interferon -15 [FII-15], e proteína hipotética de Homo sapiens MGC5566) e dois genes hipoexpressos (queratina 2A [KRT2A] e desmocolina-1 [DSC-1]). Os autores concluíram que a tecnologia de *microarray* fornece um conjunto de genes candidatos que podem servir futuramente como novos pontos de intervenção terapêutica e diagnóstico mais preciso, além de ser útil em procedimentos de triagem de indivíduos de alto risco. Outro estudo que utilizou a técnica de *microarray* determinou após a validação dos resultados a hiperexpressão dos genes interleucina-12A (IL-12A), versican (CSPG-2), MMP-1, proteína da região crítica da síndrome de Down -1 (DSCR-1), Proteína Inflamatória do Macrófago-2β (CXCL-3), proteína inibidora de apoptose -1 (BIRC-1), Cluster do antígeno de diferenciação 38 (CD38), sinalizador da proteína G reguladora-1 (RGS-1), e *Finkel-Biskis-Jinkins* oncogene do vírus murino osteossarcoma (C-FOS) em sítios gengivais com periodontite crônica severa após tratamento em relação aos controles saudáveis [8]. Os 5% genes menos expressos foram: receptor-interagindo serina/ treonina quinase-2 (RIP-2), componente do complemento 3 (C3), prostaglandina-endoperóxido sintase-2 (COX-2), interleucina-8 (IL-8), endotelina-1 (EDN-1), inibidor do ativador de plasminogênio tipo 2 (PAI-2), metaloproteinase de matriz -14 (MMP-14) e fator de regulação do Interferon -7 (IRF-7). Os autores concluíram pelo perfil de expressão gênica em tecidos periodontais após tratamento, que há ativação de vias que regulam o dano tecidual e também o reparo [8].

Considerando a investigação da expressão gênica somente envolvendo o DM2 por *microarray* (seguido do RT-qPCR), foram verificados genes diferencialmente expressos no tecido adiposo (fator de crescimento como insulina-2 [*IGF2*], receptor único C relacionado à RAR [*RORC*] e proteína tirosina fosfatase de tipo não-receptor 1 [*PTPN1*]), e também o gene *quinase piruvato tipo-M2* [*PKM2*] no tecido muscular esquelético de pacientes com DM2 [121]. Outro estudo que também utilizou a técnica de *microarray* enfocou as mudanças na expressão gênica de pacientes com DM2, os quais foram induzidos a manifestar emoções positivas (como o riso) [41]. Foi encontrada a expressão diferencial de genes com diversas funcionalidades, como a resposta imune (*PRF1, GZMM, TMSB4X* e *CD74*), transdução de sinal (*AKT2, GNL1, PDAP1* e *ADCY7*), ciclo e adesão celular, metabolismo e apoptose [41].

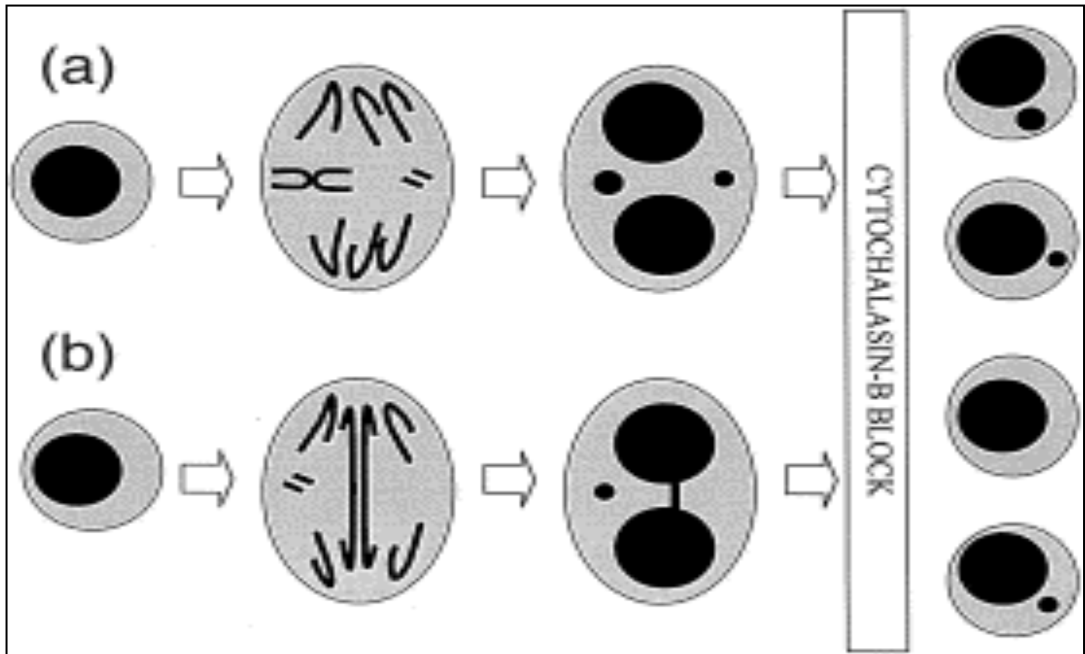
Com relação à expressão de genes do sistema imune relacionados à dislipidemia, pouca informação sobre este tema foi encontrado na literatura. Demonstrou-se infiltração aumentada de macrófagos no tecido adiposo, e esses macrófagos se mostraram ser um importante componente para a geração de resposta inflamatória crônica que ocorre nos indivíduos obesos. Tem-se observado a ativação crônica da via pró-inflamatória NF-kB e/ou da via JNK1 em tecidos alvo da insulina (adiposo, fígado e músculo) [115]. O adipócito pode ser a fonte de um grande número de citocinas/quimiocinas (TNF-α, IL-6, IL-1, proteína quimiotática de monócitos-1, etc) e adipocinas (leptina, adiponectina, e resistina) [93]. Os adipócitos também expressam receptores de várias moléculas pró-inflamatórias indicando que tais células tanto se apresentam como fonte quanto alvo de sinais pro-inflamatórios [24, 94]. Apesar dos estudos já realizados na área, a contribuição do tecido adiposo como uma fonte sistêmica de citocinas/quimiocinas circulantes ainda precisa ser melhor esclarecida [11].

A produção alterada de moléculas pró-inflamatórias (as chamadas "adipocinas") pelo tecido adiposo tem sido implicada nas complicações metabólicas da obesidade. Comparado com o tecido adiposo de indivíduos magros, tecido adiposo de obesos expressa aumento da quantidade de proteínas pró-inflamatórias como TNF-α, IL-6, iNOS (também conhecido como NOS2), TGF-β1, a proteína C-reativa, ICAM solúvel e proteína quimiotática de monócitos-1 (MCP-1) [28, 44, 85, 100, 102, 111, 112, 116]. Também foi demonstrada expressão de proteínas pró-coagulantes, tais como inibidor do ativador plasminogênio tecidual do tipo-1 (PAI-1), fator VII [20, 98, 99]. Moléculas pró-inflamatórias têm efeitos diretos sobre o metabolismo celular, como a TNF-α que diretamente diminui a sensibilidade à insulina e aumenta a lipólise nos adipócitos [45, 124]. IL-6 leva à hipertrigliceridemia in vivo, estimulando a lipólise e secreção hepática de triglicérides [79]. Foi observado em cultura de células sob estímulo de LPS de *Aggregatibacter actinomycetemcomitans*, onde foram quantificados os níveis de adiponectina (citocina derivada de adipócitos) que esta atuou como potente inibidor de osteoclastos via TLR4 (receptor semelhante a *toll*) mediada por RANKL (ligante do receptor ativador do NF-κB) [119].

Como os mecanismos envolvidos na patogênese do DM2, dislipidemia e periodontite crônica estão intrinsecamente relacionados com o estresse oxidativo, e que isso pode levar a lesões no DNA, um dos métodos mais consagrados para essa avaliação é o teste do Micronúcleo (MN). O teste do micronúcleo é um método para analisar in vivo danos nos cromossomos, e tem sido usado por muitos pesquisadores para testar a integridade do DNA após exposição celular às radiações, substâncias tóxicas e outras situções de estresse. O teste é baseado na identificação de um núcleo secundário (micronúcleo) formado em decorrência de quebras cromossômicas que não puderam ser reparadas; assim tais fragmentos cromossômicos são organizados dentro de um

micronúcleo [66] (Figura 1). Pesquisando a literatura científica, observaram-se poucos estudos que investigaram, por meio do teste do micronúcleo, lesões no DNA em pacientes com diabetes, demonstrando estes, aumento da frequencia de micronúcleos em pacientes diabéticos [70, 127]. No entanto, os próprios autores comentam a necessidade de se realizarem mais estudos para validação dos resultados.

Figura 1 - Diagrama esquemático ilustrando a origem de micronúcleos a partir de um fragmento de cromossomo acêntrico na divisão celular (A) e a origem de pontes nucleoplasmáticas a partir de pontes dicêntricas em uma célula binucleadas (B) (figura reproduzida de FENECH, 1997 [25]).



Não foi encontrado na literatura científica nenhum estudo investigando simultaneamente a expressão genética, pela técnica do *microarray*, em pacientes com DM2, dislipidemia e periodontite crônica. Além disso, não se observou nenhum estudo que buscasse correlacionar a frequência de micronúcleos com dados bioquímicos dos pacientes com uma ou mais dessas citadas patologias; além de outros dados importantes como circunferência abdominal, proporção cintura/quadril e índice de massa corporal (IMC). Dentro desse contexto, foi proposto realizar esta pesquisa para potencialmente

contribuir para a descoberta de novos genes candidatos a marcadores dessas doenças, o que poderá ser útil para o diagnóstico ou para o acompanhamento de pacientes. Também, no futuro, tais genes identificados por *microarray* como diferencialmente expressos podem potencialmente ser investigados como novos alvos terapêuticos para seletivamente regular tais mencionadas doenças.

2 PROPOSIÇÃO

Objetivo geral

O presente estudo teve por objetivo avaliar a expressão gênica de indivíduos portadores de Diabetes Mellitus tipo 2 (compensados e não compensados metabolicamente), dislipidemia e/ou periodontite crônica, além de avaliar se tais alterações metabólicas apresentam efeito mutagênico.

Objetivos específicos

(I) Nos pacientes portadores das citadas patologias, objetiva-se investigar por meio do teste do Micronúcleo (Apêndice 1) a frequência de lesões irreversíveis no DNA e correlacionar os achados de micronúcleo com os fenótipos de cada grupo de indivíduos.

(II) Objetiva-se avaliar pela técnica de *microarray* a expressão gênica no PBMC (Apêndice 1) de indivíduos portadores das citadas patologias, e validar por RT-qPCR 4 a 5 genes diferencialmente expressos (hipo ou hiperexpressos), além de correlacionar os achados de expressão gênica com os fenótipos de cada grupo de indivíduos.

3 CAPÍTULOS

3.1 Capítulo 1

Elevated micronuclei frequency in patients with type 2 diabetes, dyslipidemia and periodontitis*

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Elevated micronuclei frequency in patients with type 2 diabetes, dyslipidemia and periodontitis

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Running title: Micronuclei frequency in diabetic patients with dyslipidemia and periodontitis

Key Words: Type 2 diabetes mellitus; periodontal diseases; dyslipidemias; micronuclei; DNA damage

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Abstract

Background and Objective: The over-production of reactive oxygen species (ROS) can cause oxidative damage to a large number of molecules, including DNA, and has been associated with the pathogenesis of several disorders, such as diabetes mellitus (DM), dyslipidemia and periodontitis. We hypothesize that the presence of these diseases could proportionally increase the DNA damage. The aim of this study was to assess the micronuclei frequency, as a biomarker for DNA damage, in individuals with type 2 diabetes, dyslipidemia and periodontitis.

Materials and Methods: One hundred and fifty patients were divided into five groups based upon diabetic, dyslipidemic and periodontal status (group 1 – poor controlled diabetes with dyslipidemia and periodontal disease; group 2 – well-controlled diabetes with dyslipidemia and periodontal disease; group 3 – without diabetes with dyslipidemia and periodontal disease; group 4 – without diabetes, without dyslipidemia and with periodontal disease; and group 5 – without diabetes, dyslipidemia and periodontal disease). Blood analyses were carried out for fasting plasma glucose, HbA1c, and lipid profile. Periodontal examinations were performed, and venous blood was collected and processed for micronuclei assay. The frequency of micronuclei was evaluated by cell culture cytokinesis-block micronucleus assay. The general characteristics of each group were described by the mean and standard deviation and the data were submitted to the Mann-Whitney, Kruskal-Wallis and Spearman tests.

Results: The groups 1, 2 and 3 were similarly dyslipidemic presenting increased levels of total cholesterol, LDL cholesterol and triglycerides. Periodontal tissue destruction and local inflammation were significantly more severe in diabetics, particularly in group 1. Highest micronuclei frequency was found for well-controlled diabetics with dyslipidemia and periodontitis, and significantly higher micronuclei frequencies were

also found in patients affected by any of the systemic diseases in comparison with those systemically healthy.

Conclusions: Elevated frequency of micronuclei was found in patients affected by type 2 diabetes, dyslipidemia and periodontitis. This result suggests that these three pathologies occurring simultaneously promote an additional role to produce DNA impairment. In addition, the micronuclei assay was useful as a biomarker for DNA damage in individuals with chronic degenerative diseases.

Introduction

Diabetes mellitus (DM) comprises a group of metabolic diseases characterized by hyperglycemia resulting from defects in secretion and/or in the action of insulin. Chronic hyperglycemia in DM is associated with injuries, dysfunctions and failures of various organs in the long term, especially the eyes, kidneys, nerves, heart, and blood vessels (1,2). The prevalence of DM increases worldwide, being estimated that more than 300 million individuals will be affected by the year of 2030 (3). Type 2 Diabetes mellitus is the most common form of DM constituting 90-95% of total diabetic cases. The rest of cases comprise of type 1 diabetes and other minor forms. A factor associated with complications of DM is the process of non-enzymatic glycation of proteins, lipids and nucleic acids, with subsequent formation of advanced glycation end products (AGEs) (4). One of the main consequences of this adverse action is the formation of oxidative stress. Oxidative stress induces cellular damage and insulin resistance, and emerges as the major mechanisms for related co-morbidities (5-7). In addition, patients with type 2 diabetes present a deficient antioxidant defense compared to normal individuals (8,9). The increased production of the ROS has also been assigned to the glycation of proteins (10,11) and/or auto-oxidation of glucose during the process of hyperglycemia (12). Therefore, besides to the AGEs, the oxidative stress presents an important role in the pathogenesis and complications of DM (13).

In individuals with DM, besides the changes related to hyperglycemia, they can also be affected by dyslipidemia, which includes qualitative and quantitative changes of lipoproteins and lipid metabolism disorders (14,15). Effects of the dyslipidemic microenvironment can be altered in DM due to the presence of AGEs, which can act as co-stimulators of the cells. The hyperglycemia associated to uncontrolled DM increases the formation of AGEs, including LDL-AGE, and is associated with the largest amount

of LDL-oxidized. Both AGEs and LDL-oxidized, present at the same time in the blood of patients with DM, have several biological effects on lymphocytes and monocytes. These cells are involved in the pathogenesis of various diseases associated with DM, such as lower resistance to infections, vascular disease and greater severity of the periodontitis (16).

Periodontitis can be recognized as the sixth largest complication associated with the DM (17), as a disease to a greater extent and severity compared to individuals without DM (18-20). Periodontitis is an infectious disease that affects dental supporting tissues, caused by predominantly Gram-negative, anaerobic bacteria, such as *Porphyromonas gingivalis* and *Prevotella intermedia*. This disease induces local and systemic elevations of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 beta (IL-1β) and IL-6 (21). These molecules contribute to soft and hard periodontal tissue destruction, dental mobility, and the loss of dental elements. In regard to periodontitis, the generation of ROS is an important mechanism during phagocytosis as part of the bactericidal reaction (22-24). It has been suggested that PMNs produce and release a large quantity of ROS, culminating in heightened oxidative damage to gingival tissue, periodontal ligament, and alveolar bone as a result of stimulation by bacterial antigens (25). ROS are active in depolymerization of extracellular matrix components, in lipid peroxidation, in oxidation of enzymes such as anti-proteases, in induction of proinflammatory cytokines, and in DNA damage (26-28).

Besides generation of reactive oxygen species (ROS) by the biological mechanisms of mitochondrial respiratory chain and polymorphonuclear (PMN) activation in inflammation (28,29), the over-production of ROS associated with the pathogenesis of several disorders (30) such as diabetes mellitus, dyslipidemia and periodontitis, can contribute to injury of the host tissue (31), significant impairment to

cell integrity (32,33) and can cause oxidative damage to a large number of molecules such as carbohydrates, lipids and DNA.

Because the oxidative stress is intrinsically related to the pathogenesis of type 2 diabetes, dyslipidemia and periodontitis, and it leads to DNA damage, it seems interesting to evaluate the occurrence of DNA and its relationship with these diseases. One of the most established methods for evaluating DNA damage is the micronucleus test. The micronucleus test analyzes *in vivo* chromosome damage, and has been used by many researchers to test the integrity of DNA after cell exposure to radiation, toxic substances and other stressful situations. The test is based on the identification of a secondary nucleus (micronucleus) formed as a result of chromosomal breaks that could not be repaired; in other words, such chromosome fragments are organized within a micronucleus (34). Few studies have investigated DNA damage by micronucleus in patients with type 2 diabetes, and these studies have shown increased frequency of micronuclei in diabetic patients (35,36). Importantly, the authors themselves highlighted the need to conduct further studies to validate this finding. It is worth mentioning that it was not found any study in the literature which sought to investigate the micronucleus frequency in the context of type 2 diabetes, dyslipidemia and periodontitis, simultaneously. We hypothesize that the presence of these diseases could proportionally increase the DNA damage. Therefore, the aim of this study was to assess the micronuclei frequency as a biomarker for DNA damage in individuals affected by type 2 diabetes, dyslipidemia and periodontitis.

Materials and Methods

The present study was approved by the Ethics in Human Research Committee of the Araraquara School of Dentistry (UNESP – Univ. Estadual Paulista, Araraquara,

Brazil; Protocol number 50/06) and was conducted according to the ethical principles of the Declaration of Helsinki. All volunteers were informed about the aims and methods of this study, and they provided their written consent to participate.

Study population

Basic inclusion criteria were age from 35 to 60 years and had at least 15 natural teeth. Patients were excluded based on the following criteria: history of antibiotic therapy in the previous 3 months and/or nonsteroidal anti-inflammatory drug therapy in the previous 6 months, periodontal treatment or surgery in the preceding 6 months, pregnancy or use of contraceptives or any other hormone, current smokers or former smokers, history of anemia, use of hypolipemic drugs such as statins or fibrates, use of vitamin and/or antioxidant therapy and history of diseases that interfere with lipid metabolism, such as hypothyroidism and hypopituitarism.

Patients enrolled in this study were previously investigated regarding to MDA (malonaldehyde) and some inflammatory cytokine levels (37). A power analysis based on a pilot study determined that at least 20 patients in each group would be sufficient to assess differences in those molecules with 90% power and 95% confidence interval. To compensate possible drop-outs during the experimental period were selected 150 patients. Therefore, the population investigated here was divided into five groups of 30 patients each, based upon diabetic, dyslipidemic and periodontal status: poorly controlled diabetics with dyslipidemia and periodontitis (Group 1), well-controlled diabetics with dyslipidemia and periodontitis (Group 2), normoglycemic individuals with dyslipidemia and periodontitis (Group 3), systemically healthy individuals with periodontitis (Group 4) and systemically healthy individuals without periodontitis (Group 5).

Clinical record and physical evaluation

All participants answered a structured questionnaire about demographic characteristics, personal and family medical history, and use of medications. A trained examiner collected information from diabetic patients regarding time since diabetes onset, medication used to control hyperglicemia, and the presence of complications associated with diabetes. Subjects completed a physical examination including anthropometric data such as abdominal circumference (cm), hip (cm), waist (cm), height (m) and weight (kg). Body mass index (BMI) was evaluated.

Laboratory measurements: metabolic control and lipoprotein profile

Blood samples were collected after a 12-hour overnight fast for the evaluation of fasting plasma glucose (mg/dL) by modified Bondar & Mead method, glycated haemoglobin (HbA1c) by enzymatic immunoturbidimetry, insulin levels by the chemiluminescence method (U/L), high-sensitivity C-reactive protein by the nephelomeric method and lipid profile (total cholesterol (TC), triglycerides (TGs), and HDL) by enzymatic methods. LDL was determined by the Friedewald formula. To avoid the inclusion of individuals with transitory dyslipidemia, the cutoff points used were the highest values according to the National Cholesterol Educational Program (NCEP) Adult Treatment III (ATP III) (38): TC $\geq$ 240 mg/dL, LDL $\geq$ 160 mg/dL, HDL $\leq$ 40 mg/dL, and TGs $\geq$ 200 mg/dL. Metabolic control was considered as adequate when HbA1c < 8% and as inadequate when HbA1c $\geq$ 8%.

Periodontal clinical examination

Chronic periodontitis, as defined by the American Academy of Periodontology (39), includes local signs of inflammation and tissue destruction (presence of deep

periodontal pockets ≥ 6 mm) and loss of the connective tissue attachment of gingiva to teeth (clinical attachment loss ≥ 4mm) in at least 4 non-adjacent teeth. All patients were subjected to a periodontal clinical examination performed in six sites per tooth by a single trained calibrated examiner (A.B.S, Kappa = 0.89). Periodontal pocket depth, clinical attachment loss, and bleeding on probing were evaluated with a periodontal probe PCPUNC15-6 (Hu-Friedy®). Severe periodontal disease was defined as the presence of deep periodontal pockets ≥ 6mm with clinical attachment loss ≥ 5mm and bleeding on probing in at least 8 sites distributed in different quadrants of the dentition (40).

Blood sampling, cell culture and cytokinesis-block micronucleus (CBMN) assay

Samples of venous blood (5 mL) were collected in heparinized vacutainer tubes (Becton–Dickinson, NJ, USA) by venipuncture under sterile conditions. The samples were coded, kept in the refrigerator cooled at 4 ^{0}C in the dark and processed for micronuclei assay.

The cultures were prepared by adding 0.5 mL of isolated lymphocytes with plasma to 5 mL of complete medium containing 78% RPMI (Sigma–Aldrich Co., USA), 20% inactivated fetal bovine serum (Gibco-Invitrogen, Denmark), antibiotics (penicillin and streptomycin, Sigma– Aldrich Co., USA) and 2% phytohemagglutinin (PHA; Gibco-Invitrogen, Denmark) to stimulate cell proliferation. Cultures were incubated at 37 ^{0}C for 72 h before harvesting. Cytochalasin B (6 µg/mL, Sigma–Aldrich Co., USA) was added to the cultures 44 h after phytohemagglutinin stimulation. At 72 h, the cells were subjected to a mild hypotonic treatment (1% sodium citrate), fixed twice with methanol:acetic acid (3:1), smeared on a precleaned microscope slides and air-dried. Staining was performed with Giemsa (5% in Sorensen Buffer) to determine

the micronuclei frequency (MNF), the frequency of binucleated cells with micronuclei (MCF), the nuclear division index (NDI) and nucleoplasmatic bridges indexes, as previously described by Fenech et al. (41). NDI was calculated by scoring cells with 1, 2, 3 or 4 nuclei using the formula:

$$NDI = M1 + 2(M2) + 3(M3) + 4(M4)/N;$$

where, M1–M4 stands for the number of cells with 1–4 nuclei, and N is the total number of viable cells observed.

Statistical analysis

The distribution and normality of the variables were evaluated by the D'Agostino-Pearson test. The general characteristics of each group were described with mean and standard deviation (SD). The Mann–Whitney statistical test was used to compare the individual MCF, MNF, NDI and frequency of nucleoplasmatic bridges between the groups. The Kruskall-Wallis test, followed by Dunn's post-test was used to compare other sample characteristics, such as measurements of physical evaluation, metabolic control and lipoprotein profile. Spearman's partial correlation coefficient was used to investigate the correlations between the various parameters studied. The significance level was set at α=0.05. All analyses were carried out with GraphPad Prism software, version 5.0.

Results

Sample population

From the screened patients, 150 were identified as meeting the study inclusion criteria; of these, 30 patients assigned to 5 different groups. The general characteristics of the sample are demonstrated in Table 1. There was no statistical significant

difference among the groups with respect to gender, ethnicity and socioeconomic status. Mean age of group 5 was statistically lower than other groups. In relation to physical evaluation, the individuals of groups 1, 2 and 3 were overweight and the diabetic groups were obese and presented higher values of BMI ($p<0.05$), waist/hip proportion and abdominal circumference. The groups with diabetes had significantly increased levels of fasting glucose, HbA1c, and insulin resistance compared with nondiabetic patients. Based on ATP III (38) definition of dyslipidemia, the groups 1, 2 and 3 were similarly dyslipidemic presenting increased levels of total cholesterol, LDL cholesterol and triglycerides.

Among all diabetics, there was no difference in the age of diabetes onset. However, poorly controlled diabetics (group 1) were the most hypertensive patients and had more diabetic complications ($p<0.05$), with the most common complication being retinopathy, followed by nephropathy.

Periodontal evaluation

Table 2 showed that periodontal tissue destruction and local inflammation were significantly more severe in diabetics, particularly in group 1 (poorly controlled diabetics with dyslipidemia), which presented high index of the marginal bleeding, probing depth, clinical attachment loss and percentage of periodontal sites with suppuration. The group 2 (well-controlled diabetics with dyslipidemia) showed significant difference in relation to the presence of deeper periodontal sites (≥ 4 mm) and higher attachment loss (≥ 5 mm) when compared to groups without DM (groups 3, 4 and 5) (Table 2).

MN levels

Regarding nuclear division index (NDI), groups 1, 2 and 3 were similarly statistically higher than groups 4 and 5 (Table 3). MCF, MNF and nucleoplasmatic bridges indexes showed higher frequencies in groups 1, 2, 3 and 4 than the group 5. Concerning to the frequency of binucleated cells with micronucleus (MCF) and micronuclei frequency (MNF), group 2 showed the highest frequency of this index, being followed by groups 3 and 1. Frequencies of MCF and MNF were similarly higher in groups 2 (well-controlled diabetics with dyslipidemia and periodontitis) and 3 (normoglycemic individuals with dyslipidemia and periodontitis). Interestingly, MCF and MNF indexes of group 1 were statistically lower than group 2, indicating that the glycemic control in patients belonging to group 2 was not associated with lower DNA damage.

Correlation analyses

A correlation analysis was carried out among biochemical, periodontal and micronucleus parameters for each experimental group (1, 2, 3, 4 and 5 groups), which is showed in Table 4. Group 1 demonstrated a strong negative correlation among MNF parameter and the probing depth (mm) (-0.62), and the attachment loss (mm) (-0.60) mean. Also, group 1 showed a strong positive correlation between MNF parameter and percentage of sites with probing depth ≤ 3 mm (0.62). For group 2, moderate/strong positive correlation was observed between NDI parameter and total cholesterol (0.52), LDL cholesterol (0.54), and suppuration (0.63). Regarding group 3, it was observed moderate positive correlation between NDI parameter and percentage of periodontal sites with visible plaque (0.50). Concerning group 4, weak positive correlation was observed between the frequency of nucleoplasmatic bridges and waist/hip proportion

parameter (0.42). For group 5, it was observed moderate positive correlation between BMI (body mass index) and MCF (0.51), and MNF (0.51) parameters. Moreover, negative correlation (-0.43) was observed between both the MCF and MNF with HDL cholesterol levels.

Discussion

To the best of our knowledge, this is the first study investigating association between DNA damage by the CBMN assay and the physical examination, biochemical measurements (metabolic control and lipoprotein profile) and periodontal clinical parameters in patients who have, or not, simultaneously type 2 diabetes, dyslipidemia and periodontitis. The results presented here indicated an association between type 2 diabetes and DNA damage, regarding the frequency of binucleated cells with micronucleus (MCF), the micronuclei frequency (MNF), and nucleoplasmic bridges, especially considering the results observed for the group 2 (well-controlled diabetics with dyslipidemia and periodontitis). In the scientific literature, as regards MN assay and diabetes mellitus, Andreassi et al. (42) showed that type 2 diabetes was the major independent determinant of an increase MN frequency in circulating lymphocytes of patients with ischemic heart disease. Increase of MN frequency was also noted by Martínez-Perez et al. (35) in patients with type 2 diabetes without any microvascular or macrovascular complications. Zúñiga-González et al. (36), investigating type 1 diabetic patients, also saw a definitive increase in the MN frequency in the circumstance of uncontrolled diabetic individuals (HbA1C > 7%). Furthermore, they found a significant reduction in MN after folate supplementation for 30 days. Differently, Cinkilic et al. (43) failed to find any significant difference among MN frequency in type 1 diabetic patients, as compared to controls.

The present study unexpectedly showed higher levels of MN frequencies in well-controlled type 2 diabetic patients (Group 2) rather than in poorly controlled type 2 diabetic patients (Group 1). This result can be explained because Group 2 demonstrated the highest levels of high sensitivity C-reactive protein, triglycerides, BMI and abdominal circumference from the whole sample. C-reactive protein is a well-established marker of inflammation and cardiovascular disease (44) and can be recognized as a parameter of oxidative stress in obese subjects (45). Likewise, an increased body mass index (BMI) among overweight individuals has been shown to be associated with increased risk of DNA damage due to oxidative stress (46) and of cardiovascular disease (47,48). Unpublished results of our research group related to the same patients investigated here showed that patients of Group 2 presented high lipid peroxidation levels and high inflammatory cytokines levels (IL-1α, IL-6, IL-8, TNF-α) in plasma. These unpublished results indicated that the inflammatory and lipid peroxidation levels of the well-controlled diabetics were closer to the levels observed in the group with poorly controlled DM (group 1) when compared to the lipid peroxidation levels in the 'normoglycemic with dyslipidemia' group (group 3). Our hypothesis is that these unpublished results together with the highest levels of sensitivity C-reactive protein, triglycerides and BMI found here for group 2 could probably indicate that ROS excess related mainly with the lipid metabolic imbalance caused longer cellular effects of DNA damage. Therefore, even though Group 2 individuals get glycemic control, the DNA damage remains in the cells of patients. In addition, we consider that the lipid metabolic imbalance could explain the second higher MCF and MNF frequencies in Group 3, despite these values were not statistically different from Group 2. It could be noted that Group 3 showed the highest levels of total, HDL and LDL cholesterol (Table 1). For these parameters Group 1 showed the lowest values, as well as for MCF and

MNF. Nucleoplasmic bridges were found higher in the following groups 2, 1 and 3, as well as the triglycerides levels.

In regard to periodontal evaluation, our results show that periodontal tissue destruction and local inflammation were significantly more severe in diabetics, particularly in group 1 (poorly controlled diabetics with dyslipidemia and periodontitis). It has been reported that periodontitis is one of the first clinical manifestations of DM (49) and is recognized as a common complication in diabetic patients (17), particularly in poorly controlled diabetics (50). Periodontal destruction can be caused or enhanced by ROS and active proteases released during inflammatory and host immune responses to bacterial challenge (23,51-53). However, there has been little information regarding the role of the production of ROS by activated PMN that may affect the oxidative damage to DNA molecules of nearby cells. Here, we found that important MN indexes such as MCF, MNF and presence of nuclear bridges were statistically higher in Group 4, which presents periodontitis, than in Group 5 (totally healthy). Therefore, this is the first study to demonstrate association of the occurrence of periodontitis with DNA damage. In spite of the fact that periodontitis is a local inflammatory disease and the MN indexes were assessed systemically, this finding could be observed because of the influence of the inflammatory condition, which seems to exceed the local limits of periodontium. The premise for the interrelation between periodontitis and DM is the presence of pro-inflammatory cytokines, bacteria and toxins that are released locally on periodontal tissue and enter the systemic circulation, influencing distant tissues and organs. At the same time, the systemically pro-inflammatory cytokines involved in DM crash in periodontal tissues and aggravate the periodontal condition, resulting in a bi-directional relationship (54). Further studies are required to investigate in a larger

population, or in vitro, the DNA damage and/ or oxidative stress related with periodontal disease.

We hypothesized here that the occurrence of the type 2 diabetes, dyslipidemia and periodontitis, which are involved with oxidative-stress processes, could proportionally increase the DNA damage. The present results suggest that these three pathologies occurring simultaneously promote the raise of oxidative stress and inflammation leading to increase DNA injury. Moreover, CBMN method was useful as a biomarker for DNA damage in individuals with chronic degenerative systemic diseases such as type 2 diabetes and dyslipidemia, as well as chronic local disease, such as periodontitis. The CBMN assay is the most frequently used chromosomal biomarker in human lymphocytes to study genotoxicity and cytotoxicity both *in vitro* and *in vivo* (55,56). It is now well established that the CBMN assay in its comprehensive *cytome* mode provides concurrent information on chromosomal breakage, chromosome rearrangements and gene amplification, as well as other events, for instance cell death (both apoptosis and necrosis) and cell cytotoxicity (56). Moreover, the CBMN assay may prove to be very useful in both the prediction and possibly the clinical management of chronic degenerative diseases, including diabetes and cardiovascular disease (57).

Finally, we have shown an increase of DNA damage in type 2 diabetic patients, mainly in those patients with well-controlled T2D, under the context of the presence of dyslipidemia and periodontitis. We concluded that these three pathologies happening at the same time may present an additional role to produce DNA damage, and we could also demonstrate that the micronucleus method was useful as a biomarker for DNA damage in individuals with chronic degenerative diseases.

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Conflict of interest and sources of funding statement

The authors declare that there is no conflict of interest.

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Table 1- Characteristics of the sample: demographic, physical, biochemical and diabetic data (mean ± SD)

	GROUP 1 n=30	GROUP 2 n=30	GROUP 3 n=30	GROUP 4 n=30	GROUP 5 n=30
Gender (F / M)*	18 / 12	20 / 10	17 / 13	19 / 11	18 / 12
Age (mean ± SD)	48.0 (± 7.6)[a]	50.3 (± 6.7)[a]	49.0 (± 7.5)[a]	45.9 (± 5.9)[a]	39.3 (± 3.6)
Ethnicity (white/brown/black)	13 / 10 / 7	15 / 11 / 4	20 / 8 / 2	14 / 11 / 5	23 / 3 / 4
BMI (m/Kg^2)	30.5 (± 5.2)[a,b]	31.4 (± 4.1)[a,b]	28.4 (± 3.8)	23.7 (± 6.4)	24.5 (± 3.5)
Abdominal circunference (cm)	104.3 (± 14.6)[a]	109.3 (± 10.8)[a,b,c]	98.1 (± 9.9)[a]	98.2 (± 16.9)	87.5 (± 10.6)
Fasting glucose (mg/dl)	226.6 (± 74.2)[a,b,c,d]	137.5 (± 41.4)[b,c]	90.0 (± 6.4)	90.8 (± 7.3)	85.9 (± 6.5)
HbA_{1c} (%)	10.4 (± 1.9)[a,b,c,d]	6.6 (± 0.9)[a,b,c]	5.4 (± 0.6)	5.1 (± 0.6)	5.4 (± 0.21)
Insulin (U/L)	19.7 (± 20.9)[a,b]	21.1 (± 21.5)[a,b]	12.6 (± 8.5)	11.1 (± 12.7)	7.1 (± 4.3)
Total cholesterol (mg/dl)	242.7 (± 37.8)[a,b]	243.4 (± 42.9)[a,b]	246.1 (± 42.3)[a,b]	171.6 (± 18.5)	180.3 (± 21.5)
HDL cholesterol (mg/dl)	44.8 (± 9.5)	46.1 (± 10.5)	50.7 (± 11.1)	48.4 (± 12.6)	49.3 (± 10.1)
LDL cholesterol (mg/dl)	153.4 (± 37.0)[a,b]	147.3 (± 44.3)[a,b]	156.4 (± 44.1)[a,b]	103.8 (± 17.4)	113.5 (± 18.1)
Tryglicerides (mg/dl)	216.9 (± 94.6)[a,b]	249.8 (± 104.1)[a,b]	194.1 (± 80.6)[a,b]	93.9 (± 35.9)	87.4 (± 27.6)
Hs C-Reactive Protein	0.5 (± 0.5)	0.7 (± 0.6)[a,b]	0.4 (± 0.4)	0.4 (± 0.6)	0.3 (± 0.5)
Time since DM onset (years) (mean±SD)	6.2 (± 4.2)	5.2 (± 6.6)	-	-	-
Presence of diabetes Complications	19[e]	12	-	-	-
One	09	09	-	-	-
More than one	10	03	-	-	-
Medication for DM control:					
Hypoglycemic	20	15	-	-	-
Insulin	1	1	-	-	-
Hypoglycemic/Insulin	8	5	-	-	-
None	1	9	-	-	-

· ns-no significant difference (Kruskal-Wallis test; α=5%);

[a] p<0.05 in relation to group 5; [b] p<0.05 in relation to group 4; [c] p<0.05 in relation to group 3; [d] p<0.05 in relation to group 2 (Kruskal-Wallis test, α=5%);

[e] p<0.05 in relation to group 2, to access differences regarding complications of Diabetes (Mann-Whitney test, α=5%).

Table 2- Periodontal parameters of the sample (mean ± SD)

	GROUP 1 n=30	GROUP 2 n=30	GROUP 3 n=30	GROUP 4 n=30	GROUP 5 n=30
Number of teeth*	22.3 (± 4.2)[a]	21.6 (± 4.5)[a]	23.2 (± 3.8)[a]	24.3 (± 3.1)[a]	27.1 (± 1.8)
Percentage of sites with Visible Plaque	76.5 (± 17.4)[a,b]	69.8 (± 13.0)[a]	70.1 (± 15.7)[a]	60.8 (± 17.4)[a]	14.8 (± 6.2)
Percentage of sites with Marginal Bleeding	60.9 (± 15.2)[a,b,c,d]	46.9 (± 15.9)[a]	40.4 (± 14.8)[a]	41.3 (±12.9)[a]	8.7 (± 4.8)
Percentage of sites with Bleeding on Probing	69.3 (± 12.8)[a,b,c,d]	53.9 (± 13.8)[a]	53.0 (± 13.7)[a]	51.4 (± 13.2)[a]	12.7 (± 5.6)
Mean of the Probing Depht (mm)	4.1 (± 0.5)[a,b,c,d]	3.7 (± 0.6)[a]	3.4 (± 0.5)[a]	3.7 (± 0.4)[a]	2.1 (± 0.2)
Percentage of sites with Probing Depth ≤ 3mm	43.3 (± 14.8)[a,b,c,d]	57.0 (± 15.0)[a]	61.9 (± 14.0)[a]	53.3 (± 12.5)[a]	98.8 (± 1.5)
Percentage of sites with Probing Depth = 4-5mm	31.9 (± 11.6)[a,b]	31.0 (± 11.0)[a,b]	31.0 (± 10.6)[a,b]	41.0 (± 10.0)[a]	1.2 (± 1.5)
Percentage of sites with Probing Depth ≥ 6mm	24.8 (± 15.9)[a,b,c,d]	12.0 (± 10.8)[a,c]	7.0 (± 10.6)[a]	5.7 (± 5.9)[a]	0.0 (± 0.0)
Mean of the Attachment Loss (mm)	4.4 (± 0.7)[a,b,c,d]	3.9 (± 0.7)[a]	3.6 (± 0.5)[a]	3.8 (± 0.4)[a]	2.2 (± 0.2)
Percentage of sites with Attachment Loss ≤ 2mm	13.1 (± 8.8)[a,c]	16.5 (± 15.1)[a]	24.8 (± 16.2)[a,b]	10.3 (± 10.0)[a]	64.5 (± 13.9)
Percentage of sites with Attachment Loss = 3-4mm	39.8 (± 15.3)[b,c]	48.8 (± 14.2)[a,b]	51.6 (± 10.7)[a]	61.3 (± 10.0)[a,c]	35.5 (± 13.9)
Percentage of sites with Attachment Loss ≥ 5mm	47.1 (± 16.2)[a,b,c,d]	34.7 (± 17.6)[a,c]	23.8 (± 14.0)[a]	28.4 (± 10.5)[a]	0.0 (± 0.0)
Number of sites with Suppuration	6.8 (± 7.0)[a,b,c,d]	4.0 (± 3.3)[a]	2.0 (± 3.0)	2.0 (± 3.7)	0.0 (± 0.0)

·ns-no significant difference (Kruskal Wallis test; α=5%); [a] p<0.05 in relation to group 5; [b] p<0.05 in relation to group 4; [c] p<0.05 in relation to group 3; [d] p<0.05
in relation to group 2 (Kruskal-Wallis test, α=5%).

Table 3- Parameters evaluated in the Micronucleus assay (mean ± SD)

GROUPS	NDI Mean ± SD	MCF Mean ± SD	MNF Mean ± SD	BRIGDES Mean ± SD
GROUP 1	2.02 ± 0.19[a,b]	4.57 ± 3.0[a,c]	5.42 ± 3.80[a,b,c]	2.21 ± 1.34[a,b]
GROUP 2	1.93 ± 0.17[a,b]	7.27 ± 4.61[a,b]	8.17 ± 5.41[a,b]	2.62 ± 2.06[a,b]
GROUP 3	2.03 ± 0.19[a,b]	5.34 ± 2.81[a,b]	5.96 ± 3.63[a,b]	1.96 ± 1.23[a,b]
GROUP 4	1.85 ± 0.19	3.03 ± 0.96[a]	3.30 ± 1.34[a]	1.20 ± 0.96[a]
GROUP 5	1.82 ± 0.10	1.53 ± 0.69	1.57 ± 0.79	0.14 ± 0.35

NDI: nuclear division index; MCF: frequency of bi-nucleated cells with micronucleus; MNF: micronuclei frequency; BRIDGES: frequency of nucleoplasmatic bridges; SD = standart deviation; [a] $p<0.05$ in relation to group 5; [b] $p<0.05$ in relation to group 4; [c] $p<0.05$ in relation to group 2 (Mann-Whitney test, $\alpha=5\%$).

Table 4- Correlation coefficients among biochemical, periodontal and micronucleus assay parameters in all groups

Groups/Parameters	NDI	MCF	MNF	BRIDGES
Group 1				
Fasting glucose (mg/dl)	ns	ns	ns	0.42*
Mean of the probing depth (mm)	ns	-0.59*	-0.62*	ns
Mean of the Attachment Loss (mm)	ns	-0.57*	-0.60*	ns
Percentage of sites with Probing Depth ≤ 3mm	ns	0.58*	0.62*	ns
Percentage of sites with Probing Depth ≥ 6mm	ns	ns	-0.39*	ns
Percentage of sites with Attachment Loss = 3-4mm	ns	ns	0.38*	ns
Percentage of sites with Attachment Loss ≥ 5mm	ns	-0.48*	-0.51*	ns
Group 2				
Waist/hip proportion (cm)	ns	ns	ns	0.47*
Total Cholesterol (mg/dl)	0.52*	ns	ns	ns
LDL Cholesterol (mg/dl)	0.54*	ns	ns	ns
Percentage of sites with Probing Depth 4-5mm	-0.47*	ns	ns	ns
Number of sites with Suppuration	0.63*	ns	ns	ns
Group 3				
Weight (Kg)	ns	-0.38*	-0.41*	ns
BMI (m/Kg^2)	ns	-0.40*	-0.40*	ns
Abdominal circumference (cm)	ns	ns	ns	0.39*
Percentage of sites with Visible Plaque	0.50*	ns	ns	ns
Percentage of sites with Bleeding on Probing	ns	0.38*	ns	ns
Number of sites with Suppuration	ns	0.38*	0.42*	ns

Group 4				
Waist/hip proportion (cm)	ns	ns	ns	0.42*
Percentage of sites with Probing Depth ≤ 3mm	0.37*	ns	ns	ns
Group 5				
Number of teeth	ns	ns	ns	-0.40*
Weight (Kg)	ns	0.47*	0.47*	ns
BMI (m/Kg^2)	ns	0.51*	0.51*	ns
Abdominal circumference (cm)	ns	0.40*	0.40*	ns
HDL Cholesterol (mg/dl)	ns	-0.43*	-0.43*	ns
Tryglicerides (mg/dl)	ns	0.38*	0.38*	ns

NDI: nuclear division index; MCF: frequency of bi-nucleated cells with micronucleus; MNF: micronuclei frequency; BRIDGES: frequency of nucleoplasmatic bridges; ns-no significant difference; $^*p<0.05$ (rs, Spearman`s rank correlation coefficient; α=5%)

3.2 Capítulo 2

Genome-based expression profiling in poorly/well-controlled type 2 diabetes or normoglycemic patients with dyslipidemia and periodontitis*

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Genome-based expression profiling in poorly/well-controlled type 2 diabetes or normoglycemic patients with dyslipidemia and periodontitis

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Running title: Gene expression in diabetic patients with dyslipidemia and periodontitis

Key Words: Type 2 diabetes mellitus; periodontal diseases; dyslipidemias; inflammation; gene expression; microarray

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Abstract

Background and Objective: The type 2 diabetes mellitus (T2D), dyslipidemia and periodontitis are intrinsically interrelated pathologies. In literature, it was not observed any study which sought to investigate the blood cell gene expression profiling in individuals presenting these three diseases simultaneously. We hypothesize that the T2D, as well as the glycemic control could influence gene expression under the context of the presence of dyslipidemia and periodontitis. The aim of this study was to identify, by microarray analysis, differentially expressed genes in subjects with poorly/well-controlled T2D and normoglycemic individuals, in the circumstance of the presence of dyslipidemia and periodontitis.

Materials and Methods: Ninety (90) patients were divided into three groups of 30 patients each, based upon diabetic, dyslipidemic and periodontal status: poorly controlled diabetics with dyslipidemia and periodontitis (Group 1), well-controlled diabetics with dyslipidemia and periodontitis (Group 2), normoglycemic individuals with dyslipidemia and periodontitis (Group 3). Blood analyses were carried out for fasting plasma glucose, HbA1c, and lipid profile. Periodontal examinations were performed, and venous blood was collected and processed for gene expression evaluation by microarray. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was utilized to validate the microarray results. The general characteristics of each group were described by the mean and standard deviation and the gene expression data were submitted to bioinformatics and statistical analyses.

Results: Considering all diabetic patients, there was no difference in the age of diabetes onset, and the poorly controlled diabetics group had more diabetic complications. Periodontal tissue destruction and local inflammation were significantly more severe in diabetics, particularly in group 1. Expressed transcripts of subjects from the three

experimental groups were submitted to pairwise comparisons (Group 1 + Group 2 *versus* Group 3; Group 1 *versus* Group 2; Group 1 *versus* Group 3 and Group 2 *versus* Group 3). RT-qPCR analyses confirmed the differential gene expression of the *HLA-QA1, PDCD6, TRDV3, PPAP2B, HLA-DQB1, RIN3, VCAN, PPIC* and *SLC6A13* genes in each comparison of the three groups.

Conclusions: We concluded that we have identified differentially expressed genes in PBMC of patients with poorly/well-controlled T2D also affected by dyslipidemia and periodontitis. These genes might be candidates for further investigations as potential peripheral blood markers or new therapeutic targets of the inflammatory diseases presently studied.

Introduction

Diabetes mellitus (DM) is characterized by an elevation in blood glucose concentration. The disease is progressive and associated with the development of complications, like atherosclerosis, renal and neuronal damage, and blindness [1-4]. Type 2 diabetes mellitus (T2D) is a chronic polygenic disorder designated by defects in insulin action and/or deficiencies in pancreatic insulin secretion [5, 6] and T2D is the most common form of diabetes estimated to account for 80-90% of the diabetes [7]. Furthermore, it is important to highlight that T2D occurs synergically and/or concomitantly to other systemic diseases, endangering the health status of affected individuals [8]. Recently, a population study proved that DM2 is closely associated with dyslipidemia [9, 10]. Diabetic patients have a propensity to elevated LDL/TRG (low density lipoprotein/triglycerides) densities, even when blood glucose levels are well controlled. This is significant; as studies have demonstrated [8, 11-14] that dyslipidemia could be one of the factors associated with diabetes-induced immune cell alterations.

Poor metabolic control of T2D is associated with an increased risk for coronary artery disease and infectious diseases, including periodontitis [15-18]. Periodontitis is an infectious-inflammatory disease defined by the loss of connective tissue and alveolar bone loss. Although bacterial infection is a primary cause, the disease progression depends on the production of host mediators in response to bacterial and metabolic products [19, 20]. Previous studies have suggested that periodontal infection and DM have a two-way relationship [21, 22]. Periodontitis can be recognized as the sixth largest complication associated with the DM [23], a disease with a greater extent and severity compared to individuals without DM [24-26]. On the other hand, Lalla et al. [21] (2012) reported that DM is the strongest risk factor for periodontal infection compared to the other systemic conditions such as hypertension [27].

Former studies have also been established an association between elevated serum lipids levels and periodontitis [18, 28]. Periodontitis induced by bacteremia/endotoxemia causes elevations of pro-inflammatory cytokines such as interleukin- 1 beta (IL-1b) and tumor necrosis factor-alpha (TNF-a), which have been shown to produce alterations in the metabolism of lipids leading to hyperlipidemia [8, 11]. These cytokines lead to increased mobilization of lipids from the liver and adipose tissue [28] and increase the binding of low-density lipoprotein (LDL) to the endothelium and to smooth muscles, as well as the transcription of the LDL-receptor gene [29, 30]. It is believed that these cytokines may produce an insulin resistance syndrome similar to that observed in diabetes, initiating destruction of the β-cells of the pancreas, leading to the development of DM [8, 11].

In recent years, several studies have examined the gene expression profiling to identify differentially regulated genes of local and peripheral cells in subjects with chronic inflammatory disease [31, 32]. Considering the T2D, differential gene expression was found with a number of functionalities such as the immune response, signal transduction, cell adhesion, metabolism and apoptosis [33, 34]. In relation to dyslipidemia, it was observed an overexpression of pro-inflammatory NF-kB (nuclear factor kappa B) and JNK1 (c-Jun N-terminal kinases) pathways in insulin target tissues. It was also observed an increased expression of cytokines, chemokines and adipokines [35, 36]. Investigating the gene expression in periodontitis, it was detected high levels of proteolytic enzymes and reactive oxygen species, as well as a number of cytokines, including interleukin 1 beta (IL-1b), interleukin 6 (IL-6) and tumor necrosis factor-alpha (TNF-α) [32, 37-39]. However, it was not observed any study in literature which sought to investigate the blood cell gene expression profiling, using microarray analysis, in the circumstance of T2D, dyslipidemia and periodontitis simultaneously. Also, we did not

find any study investigating whether there is influence of glycemic control in the gene expression profile.

We hypothesize that the T2D, as well as the glycemic control could influence gene expression profile under the context of the presence of the dyslipidemia and periodontitis. Therefore, the purpose of the present study was to identify, by microarray analysis, differentially expressed genes in peripheral blood mononuclear cells (PBMC) in subjects with poorly/well-controlled T2D and normoglycemic individuals, under the occurrence of dyslipidemia and periodontitis.

Materials and Methods

Study population

A simple questionnaire was initially applied to all subjects to identify patients that were eligible to be enrolled in the study, considering the inclusion/exclusion criteria. All study participants should range in age from 35 to 60 years, have at least 15 natural teeth and have similar socio-economic level. Patients were excluded based on the following criteria: history of antibiotic therapy in the previous 3 months and/or nonsteroidal anti-inflammatory drug therapy in the previous 6 months, pregnancy or use of contraceptives or any other hormone, current smokers or former smokers, history of anemia, periodontal treatment or surgery in the preceding 6 months, use of hypolipemic drugs such as statins or fibrates, and history of diseases that interfere with lipid metabolism, such as hypothyroidism and hypopituitarism.

Patients enrolled in this study were previously investigated regarding to MDA (malonaldehyde) and some inflammatory cytokine levels [40]. In that previous study, power analysis based on a pilot study determined that at least 20 patients in each group would be sufficient to assess differences in those molecules with 90% power and 95%

confidence interval. To compensate the possibility of drop-out during the experimental period, the investigational groups consisted of 30 patients each. Therefore, the population investigated here was divided into three groups of 30 patients each, based upon diabetic status: poorly controlled diabetics with dyslipidemia and periodontitis (Group 1), well-controlled diabetics with dyslipidemia and periodontitis (Group 2) and normoglycemic individuals with dyslipidemia and periodontitis (Group 3). To obtain the total casuistic of this study, we evaluated 1788 patients.

The present study was approved by the Ethics in Human Research Committee of Araraquara School of Dentistry (UNESP – Univ. Estadual Paulista, Araraquara, Brazil; Protocol number 50/06) and was conducted according to the ethical principles of the Declaration of Helsinki. All volunteers were informed about the aims and methods of this study, and they provided their written consent to participate.

Clinical record and physical evaluation

All participants answered a structured questionnaire about demographic characteristics, and a trained examiner collected information regarding time since diabetes onset, medication used to control glucose, and the presence of complications associated with diabetes. Subjects completed a physical examination including anthropometric data such as abdominal circumference (cm), hip (cm), waist (cm) and height (m), weight (kg), and body mass index (BMI).

Metabolic control and lipoprotein profile

Blood samples were collected after a 12-hour overnight fast for the evaluation of fasting plasma glucose (mg/dL) by modified Bondar & Mead method, glycated haemoglobin (HbA1c) by enzymatic immunoturbidimetry, insulin levels by the

chemiluminescence method (U/L), high-sensitivity C-reactive protein by the nephelomeric method and lipid profile [total cholesterol (TC), triglycerides (TGs), and HDL] by enzymatic methods. LDL was determined by the Friedewald formula. To avoid the inclusion of individuals with transitory dyslipidemia, the cutoff points used were the highest values according to the National Cholesterol Educational Program (NCEP) Adult Treatment III (ATP III) [41]: TC ≥ 240 mg/dL, LDL ≥ 160 mg/dL, HDL ≤ 40 mg/dL, and TGs ≥ 200 mg/dL. Metabolic control was considered as adequate when HbA1c < 8% and as inadequate when HbA1c ≥ 8%.

Periodontal clinical examination

Clinical chronic periodontal disease, as defined by the American Academy of Periodontology [42], includes local signs of inflammation and tissue destruction (presence of deep periodontal pockets ≥ 6 mm) and loss of the connective tissue attachment of gingiva to teeth (clinical attachment loss ≥ 4mm) in at least 4 non-adjacent teeth. All patients were subjected to a periodontal clinical examination performed in six sites per tooth by a single trained calibrated examiner (A.B.S, Kappa = 0.89). Periodontal pocket depth, clinical attachment loss, and bleeding on probing were evaluated with a periodontal probe PCPUNC15-6 (Hu-Friedy®). Severe periodontal disease was defined as the presence of deep periodontal pockets ≥ 6mm with clinical attachment loss ≥ 5mm and bleeding on probing in at least 8 sites distributed in different quadrants of the dentition [43].

Subjects for Oligonucleotide Microarray Analysis

For microarray investigations, 6 patients were selected from each group, considering the greater homogeneity among them of clinical periodontal, glucose and lipid parameters. The criteria for selection of patients for each study group were:

- Group 1 (poorly controlled diabetics with dyslipidemia and periodontitis) N=6: Fasting glucose between 120-230 mg/dl; HbA1c between 8.0 -12.2; total cholesterol between 250-310 mg/dl; LDL cholesterol between 140-210 mg/dl; triglycerides between 175-290 mg/dl; showing chronic periodontitis classified in a severity level of moderate to severe with probing depth (4-5 mm) ≥ 20% of the periodontal sites; clinical attachment loss (3-4 mm) ≥ 30% of the periodontal sites, with bleeding on probing ≥ 40% of the periodontal sites.
- Group 2 (well-controlled diabetics with dyslipidemia and periodontitis) N=6: Fasting glucose between 95-160 mg/dl; HbA1c between 4.7 -7.0; total cholesterol between 250-310 mg/dl; LDL cholesterol between 140-210 mg/dl; triglycerides between 175-290 mg/dl; showing chronic periodontitis classified similarly to group 1.
- Group 3 (normoglycemic individuals with dyslipidemia and periodontitis) N=6: Fasting glucose between 80-95 mg/dl; HbA1c between 4.0 -5.8; total cholesterol between 250-310 mg/dl; LDL cholesterol between 140-210 mg/dl; triglycerides between 175-290 mg/dl; showing chronic periodontitis classified similarly to group 1.

Isolation of peripheral blood mononuclear cells and RNA extraction

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly peripheral venous blood collected from each subject by centrifugation on a Ficoll-Paque

PLUS (GE Healthcare Life Sciences, Oslo, Noruega) density gradient and consecutive washings with saline (NaCl 0.9%). Total RNA was extracted using the Trizol® reagent (Invitrogen, Rockville, MD, USA) according to manufacturer's instructions. Samples containing total RNA were purified by using RNeasy kit Protection Mini Kit (Qiagen, Hilden, Germany). RNA was quantified by NanoVue Spectrophotometer (GE Healthcare Life Sciences, Oslo, Noruega) and its integrity was assessed by agarose gel electrophoresis (1%). RNA samples were used for microarray and then real time PCR analyses only if the λ(260/280) and λ(260/230) reasons were between 1.8 to 2.2. RNA samples were stored at -80° C for further analyses.

Oligonucleotide Microarray Analysis

Five hundred nanograms of RNA from 18 samples (6 samples of each group) were used for initial input of sample in GeneChip IVT Labeling kit protocol and hybridized to U133 Plus 2.0 (Affymetrix Inc., Santa Clara, CA, USA) arrays, which comprises 54.675 human transcripts. The U133 Plus 2.0 arrays were scanned twice (GeneChip Scanner 3000 7G, Affymetrix). The raw data (CEL files) were analyzed in R environment (R Development Core Team, 2013) using the Affy [44] package from Bioconductor [45] and statistical functions. Using the algorithm MAS 5 and with the perfect match - mismatch option we normalized the arrays and calculated the signal intensity and the detection (presence or absence of expression), after the detection, transcripts considered absent in all samples were removed. Them we proceed with the statistical analysis using Student’s t-test. Genes were scored as differentially regulated if they were increased or decreased ≥ 100% (fold change ≥ 2.00 and p-value ≤ 0.01). Pairwise comparisons between subjects of the three experimental groups were

performed (Group 1 + Group 2 versus Group 3; Group 1 versus Group 2; Group 1 versus Group 3 and Group 2 versus Group 3).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) Real-Time Analysis

For the reverse transcription (RT) reaction was used SuperScript III First Strand Synthesis SuperMix (Invitrogen) with 460 ng RNA in a final volume of 20 mL. The obtained complementary DNA (cDNA) was stored at -80^{0}C until performing quantitative polymerase chain reaction (qPCR) experiments.

In order to validate the differentially expressed genes obtained by the microarray analysis, RT-qPCR analysis was subsequently conducted. We chose four/five genes identified by microarray after each of the following pairwise comparisons: Group 1 + Group 2 *versus* Group 3; Group 1 *versus* Group 2; Group 1 *versus* Group 3 and Group 2 *versus* Group 3. In addition, we identified three genes which demonstrated best housekeeping expression pattern for the three groups, therefore these genes were used as endogenous controls of the reactions. The RT-qPCR analysis was performed for the three experimental groups (n=30 each group, including patients chosen for microarray analysis), and the reactions were performed in triplicate. The quantitative real-time RT-qPCR was carried out in the 7500 Real-Time PCR-System (Applied Biosystems, Foster City, CA, USA) with TaqMan® gene expression assays (Applied Biosystems). PCR cycling parameters were: 2 minutes at 50^{0}C, 10 minutes at 95^{0}C and 40 cycles of 15 seconds at 95^{0}C and 60 seconds at 60^{0}C. Validation of probes and primers, PCR conditions and relative gene expression were carried out by the 2 (-Delta Delta C (T)) method.

Statistical analysis

Microarray data were analyzed using the R statistical environment (R Development Core Team, 2007). The distribution and normality of the variables were evaluated by the D'Agostino-Pearson test. The general characteristics of each group were described with mean and standard deviation (SD). Thereafter, Mann-Whitney U test was used for pairwise comparisons between groups, as appropriate. The Kruskall-Wallis test, followed by Dunn's post-test was used to compare other sample characteristics, such as measurements of physical evaluation, metabolic control and lipoprotein profile. Spearman correlation (r) was used to investigate associations among the various parameters studied, including the gene expression of the PBMC cells. The significance level was set at $\alpha=0.05$. All analyses were carried out with GraphPad Prism software, version 5.0.

Results

Sample population

Demographic, physical and biochemical data of the sample are demonstrated in Table 1. There was no significantly statistical difference between the participants with respect to gender, ethnicity and socioeconomic status. In relation to physical evaluation, all individuals enrolled in this present study were overweight and the diabetic groups were obese and presented higher values of BMI, waist/hip proportion and abdominal circumference. Groups 1 and 2 affected by diabetes had significantly increased levels of fasting glucose, HbA1c, and insulin resistance compared with non-diabetic patients (group 3). Among all diabetics, there was no difference in the age of diabetes onset. However, poorly controlled diabetics (group 1) were the most hypertensive patients and had more diabetic complications ($p<0.05$), with the most common complication being

retinopathy, followed by nephropathy. Based on ATP III [41] definition of dyslipidemia, all groups were dyslipidemic presenting increased levels of total cholesterol, LDL cholesterol and triglycerides.

Periodontal evaluation

Periodontal tissue destruction and local inflammation were significantly more severe in diabetics, particularly in group 1 (poorly controlled diabetics with dyslipidemia), which presented high index of the marginal bleeding, probing depth, clinical attachment loss and percentage of periodontal sites with suppuration. Group 2 (well-controlled diabetics with dyslipidemia) when compared to group without DM (group 3) showed significant difference in relation to the presence of deeper periodontal sites (≥ 4 mm) and higher attachment loss (≥ 5 mm) (Table 2).

Microarray results and validation by RT-qPCR

In each pairwise comparison (Group 1 + Group 2 versus Group 3; Group 1 versus Group 2; Group 1 versus Group 3 and Group 2 versus Group 3) it was detected more than 30.000 expressed transcripts. To check the difference in gene expression between the groups, we used the Student's t-test with the exception of transcripts that presented p value ≤ 0.01 and Fold Change (FC) ≥ ± 2. Then it was obtained a list of the most differentially expressed transcripts (up/down-regulated) for each mentioned comparison between the groups (suplemmentary material). From these lists, we selected 4 to 5 genes (up or down-regulated) each group to validate the microarray results by RT-qPCR. In addition, we identified three genes which demonstrated best housekeeping expression pattern for the three groups. Therefore *ACTG1* (Hs03044422_g1), *RPL7A* (Hs00605223_g1), *GAPDH* (Hs02758991_g1) genes were used as endogenous controls of the reactions.

For validation of microarray results, the selected genes for each pairwise comparison were (Table 3):

- Group 1 + Group 2 *vs* Group 3: *HLA-DQA1* (213831_at); *SOS1* (212777_at); *HPGDS* (206726_at); *PDCD6* (222381_at);
- Group 1 *vs* Group 2: *AGBL5* (231857_s_at); *TRDV3* (216191_s_at); *PPAP2B* (212226_s_at); *LGALS12* (223828_s_at);
- Group 1 *vs* Group 3: *HLA-DQB1* (209480_at); *RIN3* (1562005_at); *VCAN* (211571_s_at); *MCM4* (212142_at); *SEC13* (239617_at);
- Group 2 *vs* Group 3: *PPIC* (204517_at); *RHOBTB2* (1554586_a_at); *PECAM1* (1559921_at); *CCL28* (224027_at); *SLC6A13* (237058_x_at);

To confirm selected microarray results using an independent technique, normalized microarray values were compared with delta CT values of expression of the same genes obtained from RT-qPCR experiments (Expression Suite *software* - Applied Biosystems, Foster City). In relation to assessment (Group 1 + Group 2) versus Group 3, the microarray analysis showed that the *HLA-DQA1* gene was upregulated in diabetics with dyslipidemia and periodontitis (Group 1 + 2) in comparison to Group 3 and *PDCD6* genes were upregulated in normoglycemic individuals with dyslipidemia and periodontitis (Group 3) compared to Groups 1 and 2 in conjunction. RT-qPCR analysis confirmed this result (*HLA-DQA1*, p=0.001 and *PDCD6*, p=0.004) (Figure 1).

Regarding to evaluation of Group 1 *versus* Group 2, the microarray analysis presented that the *TRDV3* gene was upregulated in subjects of the group 1 and in the same way that the *PPAP2B* genes was upregulated in well-controlled diabetics with dyslipidemia and periodontitis (group 2). These results were confirmed by RT-qPCR analysis (*TRDV3*, p=0.018 and *PPAP2B,* p=0.012) (Figure 2). Concerning the

comparison Group 1 *versus* Group 3, the microarray analysis revealed that the only *HLA-DQB1* gene was upregulated in subjects of the group 1 compared to group 3 and the *RIN3* and *VCAN* genes were downregulated in normoglycemic individuals with dyslipidemia and periodontitis (Group 3). RT-qPCR analysis confirmed this result (*HLA-DQB1*, p=0.02; *RIN3*, p=0.02 and *VCAN*, p=0.001) (Figure 3).

Relating to the comparison of Group 2 *versus* Group 3, the microarray analysis showed that the *PPIC* gene was upregulated in subjects of group 2 compared to group 3 and the *SLC6A13* gene was upregulated in subjects of group 3 compared to group 2. RT-qPCR analysis confirmed this result (*PPIC*, p=0.001 and *SLC6A13*, p=0.001) (Figure 4).

Correlation analyses

Correlation analysis was carried out among biochemical, periodontal parameters and gene expression for each experimental group. Group 1 demonstrated a strong negative correlation among the attachment loss (mm) (-0.60) means and attachment loss ≥ 5 mm of the periodontal site (0.51) with *SEC13* gene expression (Table 4). Also, group 1 showed a moderate positive correlation between weight (Kg) and *HLA-DQA1* gene expression (0.55); between the hip/waist proportion and *PPAP2B* gene expression (0.43) and between the C-reactive protein and *SEC13* gene expression (0.51). For group 2, moderate positive correlation was observed between fasting glucose and *PDCD6* gene expression (0.50) and between BMI (0.40) and triglycerides (0.47) with *SOS1* gene expression (Table 5). Moreover, negative correlation (-0.54) was observed between LDL cholesterol and *SOS1* gene expression. Regarding group 3, it was observed moderate positive correlation between attachment loss (mm) mean (0.44) and *VCAN* gene expression, between abdominal circumference (cm) and *SEC13* (0.49) and

SLC6A13 gene expression (0.55) (Table 6). Likewise, negative correlation (-0.40) was observed between HbA_{1c} (%) and *SEC13* gene expression.

Discussion

This is the first report which investigated the genetic expression profile in individuals simultaneously affected by three of the most common diseases: T2D, dyslipidemia and periodontitis. When we evaluated the genome-based expression profiling of (Group 1 + Group 2) *versus* Group 3, we sought to know differentially expressed genes because the influence of T2D. Then, the results of the microarray analysis were validated by RT-qPCR and demonstrated that the *HLA-DQA1* gene was upregulated in diabetics with dyslipidemia and periodontitis (Group 1 + 2) in comparison to Group 3 and *PDCD6* gene was upregulated in Group 3 compared to Groups 1 + 2. HLA (human leukocyte antigens) region is a cluster of genes located within the major histocompatibility complex (MHC) on chromosome 6p21.3 [46-48]. MHC is a region of 4 million base pairs, which contains over 100 genes characterized by high degree of allele polymorphism [49]. The human MHC is divided into three regions: class I, class II, and class III. The class I genes are located at the telomeric end of the complex. They have a single polypeptide chain containing 3 domains associated with β2-microglobuline. The class I genes classically are HLA-A, -B and -C molecules. They are expressed on the surface of almost all tissue cells and their function is to present peptide antigens to CD8+ cytotoxic T-cells. HLA class II genes are located at the centromeric end of MHC and occupy a region of 1 million base pairs [50]. Class II molecules are heterodimeric proteins consisting of heavy α-chain and lighter β-chain. Molecules are expressed on antigen presenting cells (monocytes, macrophages, B lymphocytes, dendritic cells and activated T lymphocytes). The HLA class II genes are

HLA-DR, -DQ and -DP molecules and their major function is to present peptide antigens to CD4+ T-cells. The MHC class III region contains many genes with various functions in immune system [51]. As mentioned, we validated the finding that the *HLA-DQA1* gene was upregulated in diabetics with dyslipidemia and periodontitis (Group 1 + 2) in comparison to Group 3.

The *HLA-DQA1 (Major Histocompatibility Complex, class II, DQ Alpha-1,* OMIM 146880*)* gene belongs to the HLA class II molecules which have been implicated in the pathogenesis of a number of autoimmune diseases, due to their central role in the presentation of antigenic peptides to helper T cells. This process is important in both thymic selection of the T cell repertoire and in the initiation of a T cell-mediated immune response in the periphery. The strength of T cell activation and the nature of the resulting immune response to a given (auto) antigen may be influenced by a number of factors, including (i) the density and half-life of the HLA molecules on the surface of antigen-presenting cells (APCs), (ii) the binding affinity of the HLA molecules for the (auto)antigen and (iii) the avidity of the T cell receptors (TCRs) for the HLA/(auto) antigen complex [52]. The association of HLA class II genes in type 1 diabetes mellitus pathogenesis has been reported for several ethnicities [53-55]. However, studies investigating the association of HLA class II with T2D have revealed inconsistent results, since it has been reported positive association [56], no association [57] and a weak link between HLA class II and type 2 diabetes mellitus. The association of specific HLA genotypes with T2D susceptibility/protection depends on the ethnicity and racial background of each population. In two studies, using different genotyping methods, no association was found between T2D and the HLA class II antigens (HLA-DR, HLA-DQ) in Punjabi Sikhs [58], while a positive association with *HLA-DQA* genes was reported for Belgians [59]. In Bahrainis, a population with a high prevalence of T2D, T2D

was found significantly associated with both HLA-DRB1 and HLA-DQB1 genotypes, with some alleles appearing to confer susceptibility and others playing a protective role [56]. The inconsistencies reported in these studies may be accounted for many potential factors, such as study design and sample size [60]. Considering that literature shows inconclusive findings regard to the relationship of HLA class II with T2D, the upregulation of *HLA-DQA1* gene found here in diabetics contributes to point out the real existence of an association of HLA class II with T2D. To the best of our knowledge, there is no study focusing on the expression of the *HLA-DQA1* gene with the T2D, dislipidemia and periodontitis occurring simultaneously in the same patients. More studies need to be performed to elucidate the mechanisms that may be involved in the *HLA-DQA1* gene expression with the pathophysiology of these diseases studied here.

Other microarray result also validated was the *PDCD6* gene, which was found upregulated in Group 3 compared to Groups 1 + 2. The *PDCD6* gene *(Programmed Cell Death 6,* OMIM 601057) is related with the dysregulation of the apoptosis process, contributing to the pathogenesis of several diseases, including neurodegenerative disorders, cancer, autoimmune diseases, congenital malformations and immunodeficiency. The *PDCD6* gene encodes a calcium-binding protein belonging to the penta-EF-hand protein family and is ubiquitously expressed in the body [61, 62]. Calcium binding is important for homodimerization and for conformational changes required for binding to other protein partners. *PDCD6*, (*alias*: *apoptosis-linked gene 2* - *ALG-2*) is located on chromosome 5pter–5p15.2**.** It encodes a 191-aa protein that was originally considered pro-apoptotic [62, 63]. *PDCD6* is required for T cell receptor-, glucocorticoid- [63], and FAS- [64] induced cell death. It interacts with the SH3-binding domain containing pro-apoptotic protein AIP1 (ALG-2-interacting protein-1) [65], peflin

[66], and annexin XI [67] in a Ca^{2+}-dependent way as well as with DAPK1 (death-associated protein kinase 1) [62, 68]. During FAS-induced apoptosis, *PDCD6*, which is a 22-kDa protein, is cleaved in its N-terminus to yield a 19-kDa protein and translocates from the cytoplasmic membrane to the cytosol [62, 64]. *PDCD6* gene was upregulated in lung cancer tissue indicating that may play a role in the pathology of cancer cells and/or may be a tumor marker [69]. As reviewed by Aviel-Ronen et al. [62](2008), immunohistochemical staining has revealed high expression of *PDCD6* in primary tumors compared with normal tissues of the breast, liver, and lung [69, 70]. Both nuclear and cytoplasmic overexpression have been reported for lung cancer, especially metastatic lung adenocarcinoma (ADC), indicating that it plays a role in survival pathways [69]. Aviel-Ronen et al. [62] (2008) also demonstrated that *PDCD6* is a poor prognostic factor in both early stage non-small-cell lung carcinoma (NSCLC) and ADC. HE et al. [71] (2012), confirmed that *PDCD6* gene was associated with the NSCLC in a Chinese Han population. On the other hand, downregulation of *PDCD6* gene expression was associated with ovarian cancer development [72]. Interestingly, in the present study we found for the first time a decrease in the *PDCD6* expression in subjects with T2D compared to normoglycemics, under the context of dyslipidemia and periodontitis. Further studies are necessary to understand the reasons for the lower expression of the *PDCD6* gene in these patients.

Concerning that the difference between groups 1 and 2 is that subjects of group 1 have poorly controlled diabetics, when we compared both groups we were surprised that the upregulated gene validated for group 1 was the *TRDV3* gene. Literature shows little information about this gene. The possible involvement of the *TRDV3* (*t-cell receptor delta chain variable gene cluster*; OMIM 615459) gene in immune-inflammatory processes is not understood and it was not found any study investigating

the expression of this gene in any of the three diseases that affect patients enrolled in the present study, particularly in view of the glycemic control of the T2D. There are two main mature T-cell subtypes, those expressing alpha and beta chains, and those expressing gamma and delta chains. Unlike secreted Ig molecules, T-cell receptor chains are membrane bound and act through cell-cell contact. Gamma-delta T cells may also recognize antigens directly without presentation by the major histocompatibility complex. The genes encoding the T-cell receptor delta chain are clustered within the T-cell receptor alpha chain locus on chromosome 14q11.2 [73]. *TRDV* subfamily genes were analyzed in PBMCs from patients with chronic idiopathic (immune) thrombocytopenic purpura (ITP) [74]. ITP is an autoimmune disorder in which anti-platelet antibodies induce platelet destruction due to an imbalance immune response. *TRDV1* and *TRDV2* could be detected in most samples from ITP, as well as in healthy controls; whereas *TRDV3* could be detected in only two out of 11 cases with ITP, which could be found in 90% of healthy controls (p=0.02). In conclusion, the alteration of peripheral *TRDV* repertoire pattern might play a role in the pathogenesis of immune-mediated platelet destruction in some cases with ITP. Probst et al. [75] (2004) studying the recombination signal sequences (RSS) of all mouse *TCR V*-gene segments, found that 80% of the *TRAV* (but also a minority of *TRDV*) contains a palindrome sequence (CTGCAG) in their 23-bp spacer. These sequences are specifically recognized by some nuclear proteins that are expressed by fresh thymocytes, fresh lymphocytes and tumor cells. Recombination assays on plasmid substrates in a pre-B cell line showed that RSS containing the CTGCAG sequence can impair recombination. From the protein fractions containing the CTGCAG-binding activity, three proteins were identified: G3BP1 (a nucleic-acid-binding protein with a proposed helicase activity) and two proteins from the high-

mobility group (HMG) family--HMGB2 and HMGB3. The authors hypothesize that these proteins can affect recombination at the TCR alpha delta locus [75].

The *PPAP2B (Phosphatidic acid phosphatase type 2B,* OMIM 607125*)* gene was validated in the present study as upregulated in well-controlled diabetics with dyslipidemia and periodontitis (group 2) in comparison with group 1. The *PPAP2B* gene, also called *LPP3* for *Lipid Phosphate Phosphatase 3*, belongs to the lipid phosphate phosphatases family which comprises three members: *LPP1, LPP2* and *LPP3*. *PPAP2B* codes for a membrane glycoprotein containing six transmembrane domains widely expressed in human tissues [76] that hydrolyses lipid phosphates including lysophosphatidic acid (LPA) and sphingosine 1-phosphate. Invalidation of the murine *Ppap2b* gene results in embryonic lethality due to defects in both vasculogenesis and patterning during early development [77]. LPA and sphingosine 1-phosphate are bioactive lipids that can induce proliferation, survival, and invasiveness in certain cell types and *PPAP2B* might exert an anti-tumorigenic effect by limiting LPA signalling. Overexpression of *PPAP2B* has been shown to decrease growth, survival, and tumorigenesis of ovarian cancer cells [78]. Bianchini et al. [79] (2013) revealed that adipocytic differentiation of human mesenchymal stem cells derived from adipose tissue was associated with a significant decrease in *PPAP2B* mRNA expression, suggesting that *PPAP2B* might play a role in adipogenesis. Erbilgin et al. [80] (2013), integrating human and mouse results of their study, predict that *PPAP2B* and other genes, play a causal role in the susceptibility to atherosclerosis through a part in the vasculature. This information could be connected with our study, since patients with T2D hand out several complications, including microvascular and macrovascular problems, potentially leading to atherosclerosis.

HLA-DQB1 (*Major Histocompatibilty Complex, class II, DQ Beta-1,* OMIM 604305*)* gene was validated here as upregulated in subjects of group 1 (poorly-controlled diabetics) compared to group 3 (normoglycemics). Susceptibility to some infectious diseases has a genetic association with this particular allele of MHC [81-83]. The HLA class II molecules are especially relevant to the immune response in the event of foreign antigen recognition, because they lead to CD4+ T-cell activation via T-cell receptor (TCR) by making a complex with an antigenic peptide and presenting it to T-cell [84]. Ohyama et al. [84] (1996) and Takashiba et al. [85] (1999) reported that the *HLA-DQB1* genotype is associated with early-onset periodontitis. Furthermore, the *HLA-DQB1* gene was associated with the autoimmune response against insulin-producing islet cells that leads to insulin-dependent diabetes mellitus. Forbes et al. [86] (1995) did not find direct relationship between DQB1-defined genetic susceptibility and islet cell antibodies in type 1 diabetes at diagnosis, suggesting that variations at the DQB1 locus are not linked to the expression of this autoimmune marker of beta-cell destruction. The mechanisms by which HLA-DQ molecules confer susceptibility to type 1 diabetes are not completely understood; however, the influence of environmental and immunological challenges in a genetically predisposed individual has been recognized [87]. The activation of auto reactive T cells at the pancreas level may depend on the interplay of several factors involved in inflammatory and immunological processes. Among these, candidate mechanisms may include: (i) the amount of diabetogenic peptides; (ii) the expression levels of HLA molecules on the surface of cells actively involved in the pathogenesis of the disease; (iii) the characteristics of peripheral blood cell and pancreatic b-cell-infiltrating phenotypes; (iv) the influence of the polymorphism of HLA molecules in the presentation of diabetogenic peptides; (v) the stability of the HLA/peptide/T-cell receptor complex; (vi) the amount of accessory

stimuli for T-cell activation; and (vii) the influence of cytokines on the inflammatory process [88].

Also comparing microarray results of Group 1 *versus* Group 3, *RIN3* and *VCAN* genes were validated as downregulated in normoglycemic individuals (Group 3) compared to poorly-controlled diabetics (Group 1). The *RIN3 (Ras* and *Rab* interactor 3, OMIM 610223*)* gene is a member of the RIN family of RAS effectors [89]. The *RIN3* gene displays a tissue-specific expression pattern, with highest levels restricted to mast cells. *RIN3* was an effective promoter of endogenous RAB5 activation in human mast cells. The ability of mast cells to migrate toward stem cell factor (SCF), which requires KIT recycling and prolonged signaling, was inversely correlated with *RIN3* expression. By regulating KIT response and stability, *RIN3* may play a key role in basic mast cell functions as well as pathologies involving mast cell mediated chronic inflammation and mast cell hyperproliferation [90]. Kajuho et al. [89] (2003) advised that *RIN3*, *RAB5*, and amphiphysin II form a ternary complex that is involved in the early endocytic transport pathway. Chung et al. [91] (2012) indicated the genetic polymorphisms in *RIN3* gene resulting in an increased risk of developing the Paget's disease of bone. Costa et al. [92] (2007) revealed a group of gene categories associated with transcriptional and translational regulation, among these, the *RIN3* gene, suggesting a possible role in the improvement of clinical symptoms of sickle cell anaemia patients. In literature, it was not found any information of the expression of this gene with any T2D, dyslipidemia or periodontal diseases. We also found that *VCAN* gene was upregulated in poorly-controlled diabetics (Group 1) compared to normoglycemic individuals (Group 3). *VCAN (Versican)* gene (also known as *Chondroitin Sulfate Proteoglycan 2*, *CSPG2*) encodes a chondroitin sulfate proteoglycan, which is one of the main components of the extracellular matrix (ECM), ubiquitously expressed in almost all tissues. While versican

plays a role in normal tissue development, elevated levels of versican have been reported in most malignancies to date, including nonsolid tumors, such as human acute monocytic leukemia [93-95]. Depending on the cancer type, increased versican expression occurs in either the tumor cells themselves or in the stromal cells surrounding the tumor [93, 96-98]. Emerging evidence indicates that increased versican expression is strongly associated with poor outcomes for many different cancer types [93, 99-104].

Connecting to comparison between the Group 2 *versus* Group 3, the microarray and RT-qPCR analysis displayed that the *PPIC* gene was upregulated in well-controlled diabetics with dyslipidemia and periodontitis (group 2) in comparison with group 3. In respect to the *PPIC (Peptidyl-Prolyl Isomerase C,* OMIM 123842*)* gene, also known as cyclophilin C, this gene encodes a cellular binding protein for the immunosuppressive drug cyclosporine A, which can suppress T-cell activation [105-107]. Moreover, it has been reported that the natural cellular ligand for PPIC, cyclophilin C-associated protein (CyCAP) [108] can act as a modulator of endotoxin signaling in vivo [109, 110]. Matsuo et al. [110] (2013) evaluating the gene expression of the advanced gastric carcinoma using miRNA microarray, found that *RCC2* and *PPIC* genes were actually upregulated in gastric carcinoma tissues, and therefore both were identified as possible targets of miR-29c in gastric carcinoma. Also, Obermayer et al. [111] (2013) identified an overexpression in the *PPIC* gene, appraising the novel markers for circulating tumor cells (CTCs) in patients with epithelial ovarian cancer. These facts led us to speculate that overexpression of *PPIC* gene may affect some aspects of the immune response, such as inflammation, during the occurrence of type 2 diabetes. However, to assess the function of *PPIC* gene under the context of the diabetes pathophysiology, further studies are necessary. No information was found regarding the expression of this gene with the diseases studied here. Finally, we validated that the *SLC6A13* gene was downregulated

in patients of the group 2 compared to group 3 (normoglycemics). The *SLC6A13 (Solute carrier family 6- neurotransmitter transporter, gaba-member 13,* OMIM 615097) gene (also known as *Gaba Transporter 2*, *GAT2*), encodes a major transporter for gamma-aminobutyric acid (GABA) and taurine in peripheral tissues and appears to function in maintaining plasma levels of these solutes [112]. The mammalian genome contains four genes encoding high affinity GABA transporters, and the *SLC6A13* is one of them [113, 114]. Most of the interest in these transporters has been in relation to their roles in the inactivation of GABA signaling in the central nervous system [115-117]. Outside the central nervous system, GABA uptake has been described in liver and kidney in vivo [118, 119] and in vitro [112, 120, 121]. Neither the exact cellular distribution of *SLC6A13* nor the expression levels have been determined in literature, but it is known that this gene is highly conserved among species. GABA may play a signaling role in peripheral organs, including the immune system [122, 123]. Nevertheless, the potential contribution of the *SLC6A13* gene in immune-inflammatory processes is not understood, and in the literature we did not find any study investigating either the expression of this gene in T2D, or the linking of this gene with dislipidemia and periodontitis.

Moreover, to the best of our knowledge, this report also demonstrates for the first time significant correlations between physical examination, metabolic control and periodontal clinical parameters with gene expression in normoglycemic and poorly/well-controlled T2D subjects, with dyslipidemia and periodontitis. It was found for patients from the group 1 a moderate positive correlation between the hip/waist proportion and *PPAP2B* gene expression (0.43). The reasonable explanation could be due to the fact that the hip/waist proportion can be connected with the overweight of individuals of the group 1, and the *PPAP2B* gene expression might play a role in adipogenesis and also play a causal role in the susceptibility to atherosclerosis.

Regarding to group 2, moderate positive correlation was observed between fasting glucose and *PDCD6* gene expression (0.50). The *PDCD6* gene is related with the dysregulation of the apoptosis process, contributing to the pathogenesis of several diseases, however, no study was found regarding to T2D. Concerning correlation results for group 3, it was observed moderate positive correlation between attachment loss (mm) mean (0.44) and *VCAN* gene expression. We speculate that this happens because of increasing levels of attachment loss, and it indicates damage to the extracellular matrix of the periodontium. Therefore, the higher expression of *VCAN* may indicate an attempt by the body to control this loss of periodontal tissue caused by periodontitis. Certainly, this hypothesis should be further investigated.

Finally, the present study has explored in 54.675 human transcripts which of them would differently express in each pathological condition. From the differently identified expressed genes, additional studies are necessary to comprehend the biological reasons for their association with a specific metabolic condition. Moreover, it is interesting that our findings might be investigated in larger and ethnically different populations.

We concluded that we have identified differentially expressed genes in PBMCs cells of patients with poorly/well-controlled T2D also affected by dyslipidemia and periodontitis. These genes are candidates for further investigations to evaluate whether they could be considered peripheral blood markers of the pathologies here studied. Also, in the future, these genes could be investigated as potential new therapeutic targets that can more selectively regulate the diseases here focused.

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Conflict of interest and sources of funding statement

The authors declare that there is no conflict of interest.

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Table 1- Characteristics of the sample: demographic, physical and biochemical data (mean ± SD)

	GROUP 1 n=30	**GROUP 2** n=30	**GROUP 3** n=30
Gender (F / M)*	18 / 12	20 / 10	17 / 13
Age (mean ± SD)	48.0 (± 7.6)	50.3 (± 6.7)	49.0 (± 7.5)
Ethnicity (white/brown/black)	13 / 10 / 7	15 / 11 / 4	20 / 8 / 2
BMI (m/Kg^2)	30.5 (± 5.2)	31.4 (± 4.1)	28.4 (± 3.8)
Abdominal circunference (cm)	104.3 (± 14.6)	109.3 (± 10.8)[a]	98.1 (± 9.9)
Fasting glucose (mg/dl)	226.6 (± 74.2)[a,b]	137.5 (± 41.4)[a]	90.0 (± 6.4)
HbA_{1c} (%)	10.4 (± 1.9)[a,b]	6.6 (± 0.9)[a]	5.4 (± 0.6)
Insulin (U/L)	19.7 (± 20.9)	21.1 (± 21.5)	12.6 (± 8.5)
Total cholesterol (mg/dl)	242.7 (± 37.8)	243.4 (± 42.9)	246.1 (± 42.3)
HDL cholesterol (mg/dl)	44.8 (± 9.5)	46.1 (± 10.5)	50.7 (± 11.1)
LDL cholesterol (mg/dl)	153.4 (± 37.0)	147.3 (± 44.3)	156.4 (± 44.1)
Triglycerides (mg/dl)	216.9 (± 94.6)	249.8 (± 104.1)	194.1 (± 80.6)
Hs C-Reactive Protein	0.5 (± 0.5)	0.7 (± 0.6)	0.4 (± 0.4)
Time since DM onset (years) (mean±SD)	6.2 (± 4.2)	5.2 (± 6.6)	-
Presence of diabetes Complications	19[c]	12	-
One	09	09	-
More than one	10	03	-
Medication for DM control:	20	15	-
Hypoglycemic	1	1	-
Insulin	8	5	-
Hypoglycemic / Insulin	1	9	-
None			

· ns-no significant difference (Kruskal-Wallis test; α=5%); SD = standard deviation

[a] p<0.05 in relation to group 3; [b] p<0.05 in relation to group 2 (Kruskal-Wallis test, α=5%);

[c] p<0.05 in relation to group 2, to access differences regarding complications of Diabetes (Mann-Whitney test, α=5%).

Table 2- Periodontal parameters of the sample: (mean ± SD)

	GROUP 1 n=30	GROUP 2 n=30	GROUP 3 n=30
Number of teeth*	22.3 (+ 4.2)	21.6 (+ 4.5)	23.2 (+ 3.8)
Percentage of sites with Visible Plaque	76.5 (+ 17.4)	69.8 (+ 13.0)	70.1 (+ 15.7)
Percentage of sites with Marginal Bleeding	60.9 (+ 15.2)[a,b]	46.9 (+ 15.9)	40.4 (+ 14.8)
Percentage of sites with Bleeding on Probing	69.3 (+ 12.8)[a,b]	53.9 (+ 13.8)	53.0 (+ 13.7)
Mean of the Probing Depht (mm)	4.1 (+ 0.5)[a,b]	3.7 (+ 0.6)	3.4 (+ 0.5)
Percentage of sites with Probing Depth ≤ 3mm	43.3 (+ 14.8)[a,b]	57.0 (+ 15.0)	61.9 (+ 14.0)
Percentage of sites with Probing Depth = 4-5mm	31.9 (+ 11.6)	31.0 (+ 11.0)	31.0 (+ 10.6)
Percentage of sites with Probing Depth ≥ 6mm	24.8 (+ 15.9)[a,b]	12.0 (+ 10.8)[a]	7.0 (+ 10.6)
Mean of the Attachment Loss (mm)	4.4 (+ 0.7)[a,b]	3.9 (+ 0.7)	3.6 (+ 0.5)
Percentage of sites with Attachment Loss ≤ 2mm	13.1 (+ 8.8)[a]	16.5 (+ 15.1)	24.8 (+ 16.2)
Percentage of sites with Attachment Loss = 3-4mm	39.8 (+ 15.3)[a]	48.8 (+ 14.2)	51.6 (+ 10.7)
Percentage of sites with Attachment Loss ≥ 5mm	47.1 (+ 16.2)[a,b]	34.7 (+ 17.6)a	23.8 (+ 14.0)
Number of sites with Suppuration	6.8 (+ 7.0)[a,b]	4.0 (+ 3.3)	2.0 (+ 3.0)

·ns-no significant difference (Kruskal Wallis test; α=5%);

[a] $p<0.05$ in relation to group 3; [b] $p<0.05$ in relation to group 2 (Kruskal-Wallis test, α=5%).

Table 3- Gene expression in PBMCs cells of the sample (Microarray)

Groups/Probe Set ID	Gene Title	Gene Symbol	FC	p. value
(Group 1 + 2) *vs* Group 3				
213831_at	Major Histocompatibility Complex, class II, DQ alpha 1	*HLA-DQA1*	127.80	0.002
212777_at	Son of sevenless homolog 1 (Drosophila)	*SOS1*	2.60	0.005
206726_at	Prostaglandin D2 synthase, hematopoietic	*HPGDS*	-2.18	0.002
222381_at	Aryl-hidrocarbon receptor repressor	*PDCD6*	-2.43	0.007
Group 1 *vs* Group 2				
231857_s_at	ATP/GTP binding protein-like 5	*AGBL5*	6.08	0.002
216191_s_at	T-cell receptor alpha locus	*TRDV3*	2.00	0.009
212226_s_at	Phosphatidic acid phosphatase type 2B	*PPAP2B*	-2.68	0.005
223828_s_at	Lectin, Galactoside-binding, soluble, 12 (galectin 12)	*LGALS12*	-2.09	0.007
Group 1 *vs* Group 3				
209480_at	Major Histocompatibility Complex, class II, DQ beta 1	*HLA-DQB1*	131.31	0.005
1562005_at	Ras and Rab interaction 3	*RIN3*	2.0	0.005
211571_s_at	Versican	*VCAN*	2.0	0.009
239617_at	SEC13 homolog (S. cerevisiae)	*SEC13*	2.0	0.007
212142_at	Minichromosome maintenance	*MCM4*	-4.46	0.0001
Group 2 *vs* Group 3				
204517_at	Peptidylprolyl isomerase C (cyclosphilin C)	*PPIC*	2.21	0.008
1554586_a_at	Rho-related BTB domain	*RHOBTB2*	2.13	

	containing 2			0.007
1559921_at	Platelet/endotelial cell adhesion molecule (CD31 antigen)	*PECAM1*	2.0	0.004
224027_at	Chemikine (cc-motif) ligand 28	*CCL28*	-2.0	0.006
237058_x_at	Solute carrier Family 6 (neurotransmitter transporter, GABA), member 13	*SLC6A13*	-4.00	0.003

FC – Fold Change

Table 4- Correlation among biochemical, periodontal and gene expression parameters (group 1)

Parameters	*SOS1*	*HLA-DQA1*	*PPAP2B*	*LGALS12*	*SEC13*
Weight (Kg)	-0.43*	0.55*	0.45*	ns	ns
Waist/hip proportion (cm)	ns	ns	0.43*	ns	ns
Porcentage of sites with Visible Plaque	ns	-0.52*	ns	ns	ns
HbA_{1c} (%)	ns	ns	ns	-0.45*	ns
Hs C-Reactive Protein	ns	ns	ns	ns	0.51*
Mean of the probing depth (mm)	ns	ns	ns	ns	-0.52
Mean of the Attachment Loss (mm)	ns	ns	ns	ns	-0.60*
Percentage of sites with Probing Depth ≤ 3mm	ns	ns	ns	ns	0.48*
Percentage of sites with Probing Depth ≥ 6mm	ns	ns	ns	ns	-0.48*
Percentage of sites with Attachment Loss ≥ 5mm	ns	ns	ns	ns	0.51*

ns-no significant difference; *p<0.05 (rs, Spearman`s rank correlation coefficient; α=5%)

Table 5- Correlation among biochemical, periodontal and gene expression parameters (group 2)

Parameters	*SOS1*	*HLA-DQA1*	*PPAP2B*	*LGALS12*	*PDCD6*	*PECAM1*
Fasting glucose (mg/dl)	ns	ns	ns	ns	0.50*	ns
BMI (m/Kg^2)	0.40*	ns	-0.43*	ns	ns	ns
Porcentage of sites with Visible Plaque	ns	ns	ns	ns	ns	-0.40*
LDL Cholesterol (mg/dl)	-0.54*	ns	ns	ns	ns	ns
Tryglicerides (mg/dl)	0.47*	ns	ns	ns	ns	ns
Mean of the probing depth (mm)	ns	-0.41*	ns	ns	ns	ns
Mean of the Attachment Loss (mm)	ns	-0.49*	ns	ns	ns	ns
Percentage of sites with Probing Depth ≤ 3mm	ns	0.43*	ns	ns	ns	ns
Percentage of sites with Attachment Loss ≤ 2mm	ns	ns	ns	-0.46*	ns	ns
Percentage of sites with Attachment Loss =3-4mm	ns	0.43*	ns	ns	ns	ns
Percentage of sites with Attachment Loss ≥ 5mm	ns	-0.53*	ns	ns	ns	ns

ns-no significant difference; $^*p<0.05$ (rs, Spearman`s rank correlation coefficient; α=5%)

Table 6- Correlation among biochemical, periodontal and gene expression parameters (group 3)

Parameters	*SOS1*	*HLA-DQB1*	*VCAN*	*SEC13*	*PDCD6*	*SLC6A13*	*HPGDS*
Fasting glucose (mg/dl)	ns	0.36*	ns	ns	ns	ns	0.37*
BMI (m/Kg^2)	ns	ns	ns	ns	ns	0.42*	ns
HbA$_{1c}$ (%)	ns	ns	ns	-0.40*	ns	ns	ns
Weight (Kg)	ns	ns	ns	0.36*	ns	0.42*	ns
Waist/hip proportion (cm)	ns	ns	ns	ns	ns	0.43*	ns
Abdominal circumference (cm)	ns	ns	ns	0.49*	0.37*	0.55*	ns
Insulin (U/L)	ns	ns	-0.36*	ns	ns	ns	ns
LDL Cholesterol (mg/dl)	ns	ns	ns	ns	ns	-0.40*	ns
Mean of the probing depth (mm)	ns	ns	0.36*	ns	ns	ns	ns
Mean of the Attachment Loss (mm)	0.38*	ns	0.44*	ns	ns	ns	ns

ns-no significant difference; $^*p<0.05$ (rs, Spearman`s rank correlation coefficient; α=5%)

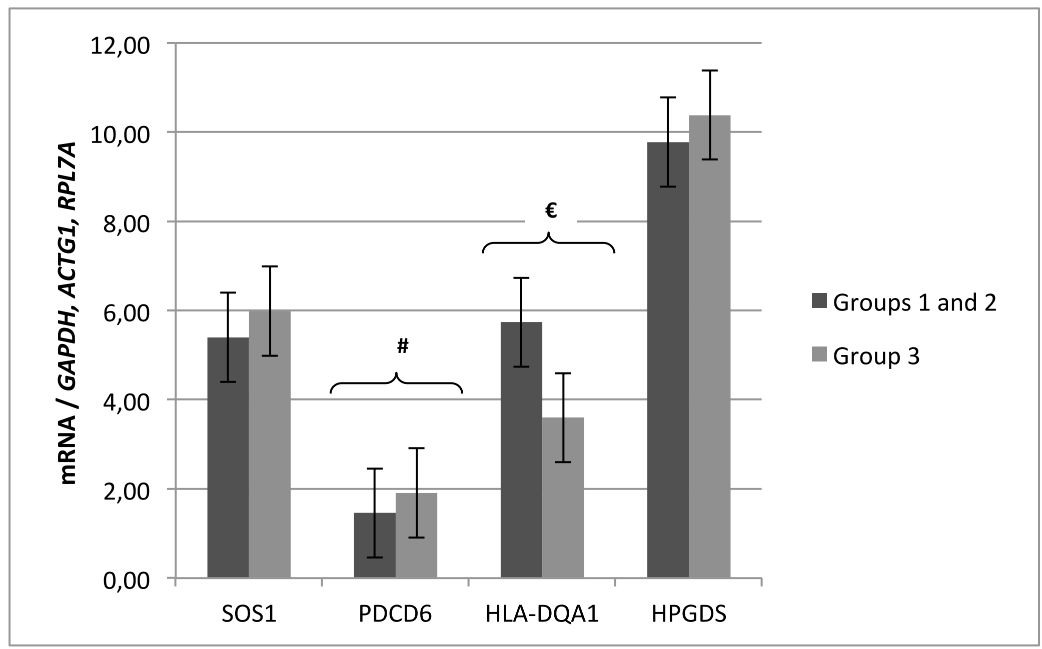

Figure 1. RTqPCR values of expression of *SOS1, PDCD6, HLA-DQA1* and *HPGDS* genes in Groups 1 + 2 *vs* Group 3. All values were normalized to *GAPDH, ACTG1* and *RPL7A* genes. Data represent the mean ± SD. # = Significant difference in *PDCD6* gene expression between Groups 1 + 2 and Group 3 (p=0.001); € = Significant difference in *HLA-DQA1* gene expression between Groups 1 + 2 and Group 3 (p=0.015). (Mann Whitney U test; α=5%)

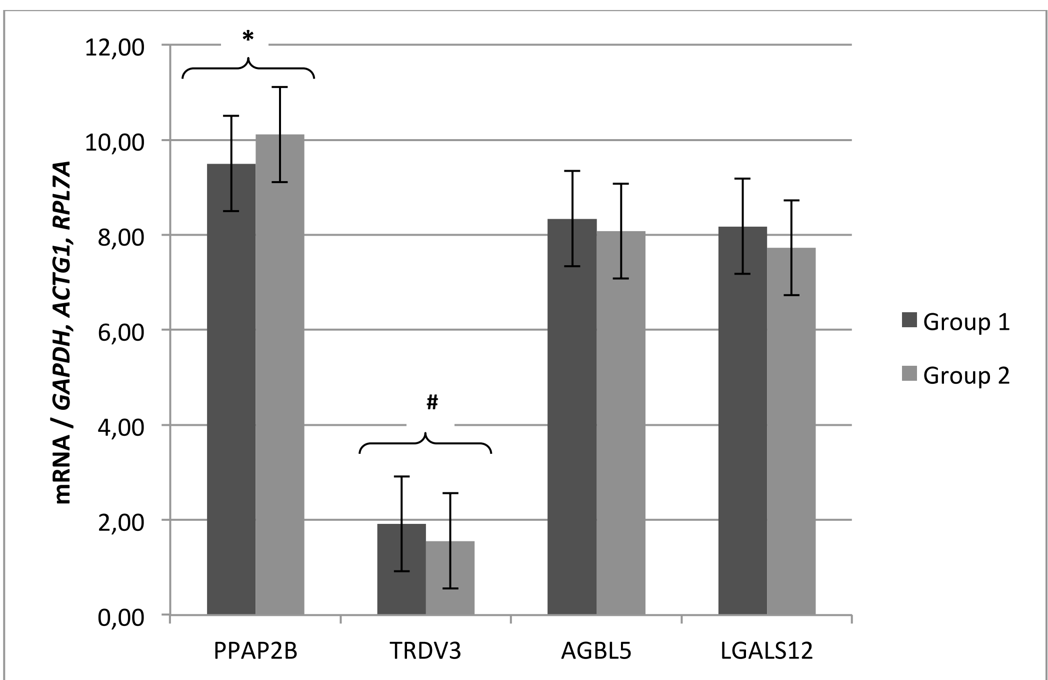

Figure 2. RTqPCR values of expression of *PPAP2B, TRDV3, AGLB5* and *LGALS12* genes in Groups 1 and 2. All values were normalized to *GAPDH, ACTG1* and *RPL7A* genes. Data represent the mean ± SD. * = Significant difference in *PPAP2B* gene expression between Groups 1 and 2 (p=0.012); # = Significant difference in *TRDV3* gene expression between Groups 1 and 2 (p=0.018). (Mann Whitney U test; α=5%)

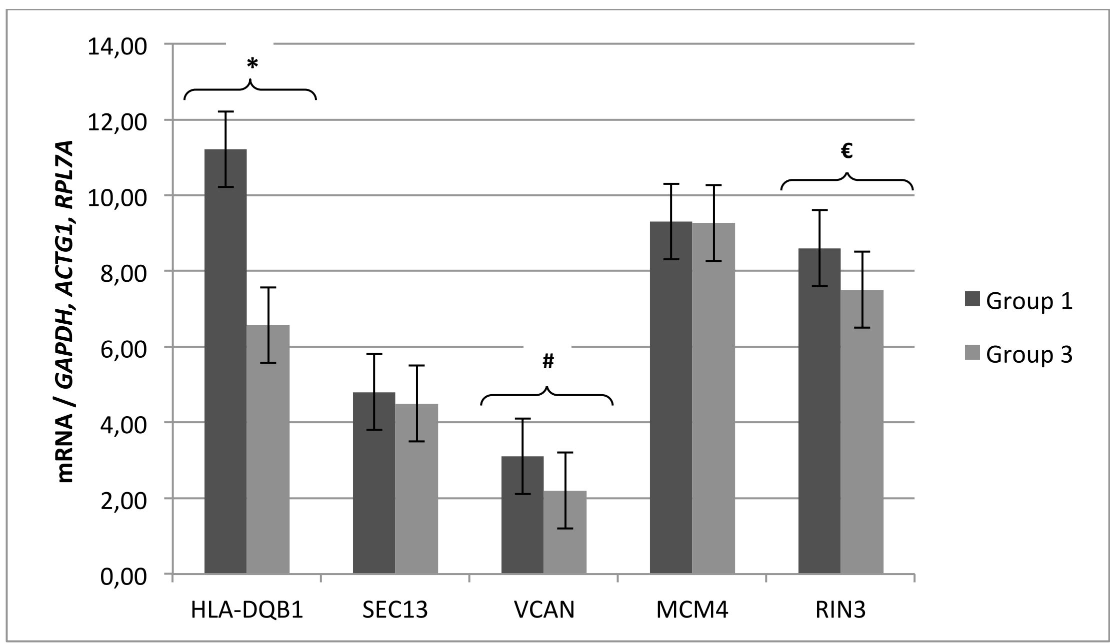

Figure 3. RTqPCR values of expression of *HLA-DQB1, SEC13, VCAN, MCM4* and *RIN3* genes in Groups 1 and 3. All values were normalized to *GAPDH, ACTG1* and *RPL7A* genes. Data represent the mean ± SD. * = Significant difference in *HLA-DQB1* gene expression between Groups 1 and 3 (p=0.01); # = Significant difference in *VCAN* gene expression between Groups 1 and 3 (p=0.001); € = Significant difference in *RIN3* gene expression between Groups 1 and 3 (p=0.02). (Mann Whitney U test; α=5%)

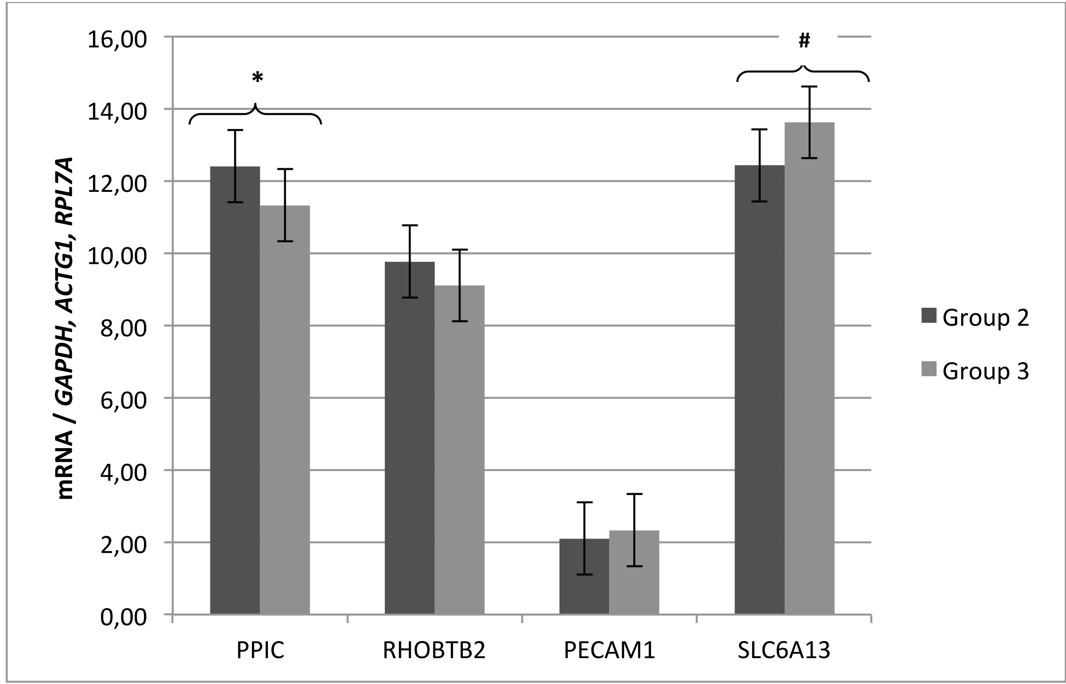

Figure 4. RTqPCR values of expression of *PPIC, RHOBTB2, PECAM1* and *SLC6A13* genes in Groups 2 and 3. All values were normalized to *GAPDH, ACTG1* and *RPL7A* genes. Data represent the mean ± SD. * = Significant difference in *PPIC* gene expression between Groups 2 and 3 (p=0.007); # = Significant difference in *SLC6A13*gene expression between Groups 2 and 3 (p=0.0001). (Mann Whitney U test; α=5%)

Supplemmentary Material

Table 1. Gene expression signature in PBMCs of the pairwise comparison (Group 1+Group 2 *versus* Group 3)

Probe Set ID	FC	p.value	Gene Title	Gene Symbol
213831_at	127,8397	0,00233	major histocompatibility complex, class II, DQ alpha 1	HLA-DQA1
209480_at	105,2995	0,002	major histocompatibility complex, class II, DQ beta 1	HLA-DQB1
236203_at	14,46415	0,00653	major histocompatibility complex, class II, DQ alpha 1	HLA-DQA1
212999_x_at	12,49405	0,00284	major histocompatibility complex, class II, DQ beta 1	hCG_1998957
220254_at	4,827312	0,00186	low density lipoprotein-related protein 12	LRP12
215449_at	3,311409	0,00944	benzodiazapine receptor (peripheral)-like 1	BZRPL1
211011_at	3,251403	0,00099	collagen, type XIX, alpha 1	COL19A1
230576_at	3,22068	0,00264	Biogenesis of lysosome-related organelles complex-1, subunit 3	BLOC1S3
201246_s_at	3,021103	0,00212	OTU domain, ubiquitin aldehyde binding 1	OTUB1
204679_at	2,897593	0,00746	potassium channel, subfamily K, member 1	KCNK1
219596_at	2,856416	0,00481	THAP domain containing 10	THAP10
208587_s_at	2,663893	0,00076	olfactory receptor, family 1, subfamily E, member 1	OR1E1
212777_at	2,601361	0,00563	son of sevenless homolog 1 (Drosophila)	SOS1
213660_s_at	2,59987	0,01031	topoisomerase (DNA) III beta	TOP3B
1553269_at	2,270608	0,00292	zinc finger protein 718	ZNF718
225784_s_at	2,267141	0,00723	KIAA1166	KIAA1166
1568665_at	2,247672	0,00046	ring finger protein 103	RNF103
1562367_at	2,182349	0,00509	chromosome 15 open reading frame 54	C15orf54
207794_at	2,034288	0,00117	chemokine (C-C motif) receptor 2 /// chemokine (C-C motif) receptor 2-like	CCR2
242003_at	1,993506	0,00557	Glutamate-rich 1	ERICH1
220058_at	1,902898	0,00282	chromosome 17 open reading frame 39	C17orf39
220064_at	1,834209	0,00763	tetratricopeptide repeat domain 21B	TTC21B
238925_at	1,769697	0,00701	syntrophin, beta 2	SNTB2
225496_s_at	1,710335	0,00516	synaptotagmin-like 2	SYTL2
1562005_at	1,665437	0,00872	Ras and Rab interactor 3	RIN3
218663_at	1,576927	0,00603	non-SMC condensin I complex, subunit G	NCAPG
212161_at	1,555851	0,01043	adaptor-related protein complex 2, alpha 2 subunit	AP2A2
217933_s_at	1,540789	0,00664	leucine aminopeptidase 3	LAP3
218999_at	1,519129	0,00404	transmembrane protein 140	TMEM140
216894_x_at	1,512364	0,00773	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	CDKN1C
208594_x_at	1,476609	0,00341	leukocyte immunoglobulin-like receptor	LILRA6
226603_at	1,437445	0,01013	sterile alpha motif domain containing 9-like	SAMD9L
222499_at	1,413399	0,00625	mitochondrial ribosomal protein S16	MRPS16
209457_at	1,411317	0,00792	dual specificity phosphatase 5	DUSP5
1553612_at	1,365605	0,00207	zinc finger protein 354B	ZNF354B

204423_at	1,359886	0,00117	muskelin 1, intracellular mediator containing kelch motifs	MKLN1
229743_at	1,353732	0,0078	zinc finger protein 438	ZNF438
224969_at	1,349789	0,00127	ataxin 7-like 3	ATXN7L3
204663_at	1,349419	0,00785	malic enzyme 3, NADP(+)-dependent, mitochondrial	ME3
225514_at	1,346615	0,00628	chromosome 14 open reading frame 21	C14orf21
231824_at	1,343731	0,00015	La ribonucleoprotein domain family, member 2	LARP2
216336_x_at	1,337576	0,00535	metallothionein 1E	MT1E
216640_s_at	1,321024	0,00648	protein disulfide isomerase family A, member 6	PDIA6
202346_at	1,302491	0,00623	ubiquitin-conjugating enzyme E2K (UBC1 homolog, yeast)	UBE2K
223393_s_at	1,289064	0,01029	teashirt zinc finger homeobox 3	TSHZ3
218589_at	1,278328	0,00441	purinergic receptor P2Y, G-protein coupled, 5	P2RY5
222978_at	1,259414	0,00088	surfeit 4	SURF4
206437_at	1,257792	0,00193	sphingosine-1-phosphate receptor 4	S1PR4
203432_at	1,255045	0,01032	thymopoietin	TMPO
205885_s_at	1,254489	0,00927	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)	ITGA4
217317_s_at	1,248773	0,00628	hect domain and RLD 2 pseudogene 3	HERC2P2
210117_at	1,233131	0,0064	sperm associated antigen 1	SPAG1
208836_at	1,230448	0,01048	ATPase, Na+/K+ transporting, beta 3 polypeptide	ATP1B3
204342_at	1,227638	0,00597	solute carrier family 25 (mitochondrial carrier; phosphate carrier)	SLC25A24
219770_at	1,225332	0,00765	glycosyltransferase-like domain containing 1	GTDC1
214700_x_at	1,21002	0,00781	RAP1 interacting factor homolog (yeast)	RIF1
207438_s_at	1,205111	0,00109	snurportin 1	SNUPN
205633_s_at	1,19814	0,00065	aminolevulinate, delta-, synthase 1	ALAS1
209103_s_at	1,19788	0,00339	ubiquitin fusion degradation 1 like (yeast)	UFD1L
220052_s_at	1,197094	0,00885	TERF1 (TRF1)-interacting nuclear factor 2	TINF2
227114_at	1,194763	0,0033	ring finger protein 214	RNF214
232652_x_at	1,175745	0,00373	SCAN domain containing 1	SCAND1
208840_s_at	1,174662	0,00462	GTPase activating protein (SH3 domain) binding protein 2	G3BP2
209229_s_at	1,172541	0,0046	SAPS domain family, member 1	SAPS1
227521_at	1,171771	0,00058	F-box protein 33	FBXO33
202375_at	1,16856	0,00696	SEC24 related gene family, member D (S. cerevisiae)	SEC24D
222411_s_at	1,158127	0,0068	signal sequence receptor, gamma (translocon-associated protein gamma)	SSR3
37577_at	1,142225	0,00634	Rho GTPase activating protein 19	ARHGAP19
201240_s_at	1,137959	0,00547	signal peptidase complex subunit 2 homolog (S. cerevisiae)	SPCS2
208680_at	1,130521	0,00685	peroxiredoxin 1	PRDX1
201964_at	1,129079	3,81E-05	senataxin	SETX
225205_at	1,126045	0,00822	kinesin family member 3B	KIF3B
201585_s_at	1,121729	0,00829	splicing factor proline/glutamine-rich	SFPQ
212733_at	1,110418	0,006	KIAA0226	KIAA0226
201086_x_at	1,076536	0,0056	SON DNA binding protein	SON

217942_at	-1,09668	0,00685	mitochondrial ribosomal protein S35	MRPS35
212539_at	-1,09698	0,00449	chromodomain helicase DNA binding protein 1-like	CHD1L
209932_s_at	-1,11405	0,00667	deoxyuridine triphosphatase	DUT
203171_s_at	-1,13516	0,00299	KIAA0409	KIAA0409
225951_s_at	-1,1715	0,00617	Chromodomain helicase DNA binding protein 2	CHD2
202867_s_at	-1,17217	0,00424	DnaJ (Hsp40) homolog, subfamily B, member 12	DNAJB12
227385_at	-1,17972	0,00731	phosphatidic acid phosphatase type 2 domain containing 2	PPAPDC2
219041_s_at	-1,18731	0,00864	replication initiator 1	REPIN1
223988_x_at	-1,20701	0,00752	methyltransferase 11 domain containing 1	LOC731602
222149_x_at	-1,24257	0,00821	golgi autoantigen, golgin subfamily a, 8A	FLJ32679
223089_at	-1,24585	0,00171	vezatin, adherens junctions transmembrane protein	VEZT
233480_at	-1,27494	0,00364	Transmembrane protein 43	TMEM43
65718_at	-1,27548	0,00432	G protein-coupled receptor 124	GPR124
225826_at	-1,28786	0,00574	methylmalonic aciduria (cobalamin deficiency) cblB type	MMAB
236721_at	-1,29637	0,00612	alkB, alkylation repair homolog 1 (E. coli)	ALKBH1
224037_at	-1,30239	0,00948	similar to PRO1405	LOC100129865
231090_s_at	-1,30316	0,00771	AT rich interactive domain 2 (ARID, RFX-like)	ARID2
239391_at	-1,30359	0,00319	Family with sequence similarity 120A opposite strand	FAM120AOS
229491_at	-1,32094	0,00291	Na+/H+ exchanger domain containing 2	NHEDC2
218808_at	-1,3217	0,00795	DALR anticodon binding domain containing 3	DALRD3
231958_at	-1,3439	0,00388	Chromosome 3 open reading frame 31	C3orf31
220936_s_at	-1,36171	0,00516	H2A histone family, member J	H2AFJ
1563090_at	-1,38407	0,00048	coiled-coil domain containing 33	CCDC33
1556277_a_at	-1,38456	0,00656	PAP associated domain containing 4	PAPD4
229693_at	-1,38718	0,00409	RIKEN cDNA A730055C05-like	LOC388335
243294_at	-1,39062	0,00962	zinc finger protein 780B	ZNF780B
203858_s_at	-1,39281	0,00438	COX10 homolog, cytochrome c oxidase assembly protein	COX10
201163_s_at	-1,39512	0,00313	insulin-like growth factor binding protein 7	IGFBP7
1558217_at	-1,40299	0,01026	schlafen family member 13	SLFN13
232486_at	-1,41069	0,00811	leucine rich repeat and fibronectin type III domain containing 1	LRFN1
220973_s_at	-1,42249	0,00131	SHANK-associated RH domain interactor	SHARPIN
1570342_at	-1,42501	0,00301	natural killer-tumor recognition sequence	NKTR
210808_s_at	-1,43418	0,00225	NADPH oxidase 1	NOX1
1561965_at	-1,43799	0,00392	Small nuclear ribonucleoprotein polypeptide B"	SNRPB2
1561190_at	-1,43887	0,00668	cyclin-dependent kinase-like 3	CDKL3
207490_at	-1,44813	0,00483	tubulin, alpha 4b (pseudogene)	TUBA4B
216583_x_at	-1,45154	0,00244	nucleolar protein family A, member 2 (H/ACA small nucleolar RNPs)	NOLA2
233376_at	-1,45338	0,00424	G protein interaction factor 2-like mRNA sequence	---
235706_at	-1,45582	0,00236	carboxypeptidase M	COM
227421_at	-1,45753	0,01001	chromosome 21 open reading frame 57	C21orf57

208368_s_at	-1,46668	0,00944	breast cancer 2, early onset	BRCA2
201162_at	-1,46782	0,00756	insulin-like growth factor binding protein 7	IGFBP7
209426_s_at	-1,49141	0,00856	alpha-methylacyl-CoA racemase	AMACR
225683_x_at	-1,49262	0,0014	phosphohistidine phosphatase 1	PHPT1
1555447_at	-1,58808	0,00905	G protein-coupled receptor 114	GPR114
200770_s_at	-1,59303	0,00485	laminin, gamma 1 (formerly LAMB2)	LAMC1
1564301_a_at	-1,62416	0,00959	RPA interacting protein	RPAIN
223137_at	-1,64991	0,00913	zinc finger, DHHC-type containing 4	ZDHHC4
210290_at	-1,65148	0,00135	zinc finger protein 174	ZNF174
1569998_at	-1,67472	0,00793	monocyte to macrophage differentiation-associated 2	MMD2
202283_at	-1,68083	0,00012	serpin peptidase inhibitor	SERPINF1
206703_at	-1,69888	0,00278	cholinergic receptor, nicotinic, beta 1 (muscle)	CHRNB1
241599_at	-1,71572	0,00455	LSM11, U7 small nuclear RNA associated	LSM11
217753_s_at	-1,72097	0,00849	ribosomal protein S26 /// similar to 40S ribosomal protein S26	LOC728937
1552812_a_at	-1,72369	0,00982	SUMO1/sentrin specific peptidase 1	SENP1
227032_at	-1,73334	0,00423	plexin A2	PLXNA2
204485_s_at	-1,74269	0,01038	target of myb1 (chicken)-like 1	TOM1L1
209160_at	-1,75357	0,00964	aldo-keto reductase family 1	AKR1C3
1569864_at	-1,75659	0,00551	serine active site containing 1	SERAC1
1553674_at	-1,76859	0,00139	leucine rich repeat containing 44	LRRC44
233301_at	-1,8011	0,00716	3-oxoacid CoA transferase 2	OXCT2
215243_s_at	-1,8016	0,00169	gap junction protein, beta 3, 31kDa	GJB3
1563969_at	-1,80255	0,01007	FLJ33360 protein	FLJ33360
238613_at	-1,80271	0,01021	sterile alpha motif and leucine zipper containing kinase AZK	ZAK
1569110_x_at	-1,85442	0,00161	programmed cell death 6 pseudogene	LOC728613
214516_at	-1,91263	0,00322	histone cluster 1, H4i	HIST1H4A
229883_at	-1,93122	0,00026	glutamate receptor, ionotropic, N-methyl D-aspartate 2D	GRIN2D
210313_at	-1,9682	0,00116	leukocyte immunoglobulin-like receptor	LILRA4
205962_at	-1,98988	0,00749	p21 (CDKN1A)-activated kinase 2	PAK2
204542_at	-2,02213	0,00648	ST6	ST6GALNAC2
216821_at	-2,05556	0,00054	keratin 8 /// similar to keratin 8 /// keratin 8 pseudogene 9	KRT8
1554176_a_at	-2,06167	0,01013	chromosome 3 open reading frame 33	C3orf33
234936_s_at	-2,10446	0,00838	coiled-coil and C2 domain containing 2A	CC2D2A
227673_at	-2,11798	0,0056	zinc ribbon domain containing 1	ZNRD1
1558557_at	-2,13613	0,00066	chromosome 16 open reading frame 62	C16orf62
206726_at	-2,18855	0,00197	prostaglandin D2 synthase, hematopoietic	PGDS
1553417_at	-2,19623	0,00026	chromosome 11 open reading frame 44	C11orf44
231172_at	-2,28869	0,00824	chromosome 9 open reading frame 117	C9orf117
1559561_at	-2,29844	0,0071	F-box protein, helicase, 18	FBXO18
234699_at	-2,33485	0,00568	ribonuclease, RNase A family, 7	RNASE7

216882_s_at	-2,35172	0,00762	nebulette	NEBL
217014_s_at	-2,41921	0,00053	alpha-2-glycoprotein 1, zinc-binding /// similar to ZN-alpha-2-glycoprotein	AZGP1
222381_at	-2,43722	0,00697	Aryl-hydrocarbon receptor repressor /// CDNA FLJ37304 fis	PDCD6
220574_at	-2,80993	0,00457	sema domain	SEMA6D
218625_at	-2,95422	0,00186	neuritin 1	NRN1
212142_at	-3,18001	0,0005	minichromosome maintenance complex component 4	MCM4

FC = Fold change

Table 2. Gene expression signature in PBMCs of the pairwise comparison (Group 1 *versus* Group 2)

Probe Set ID	FC	p.value	Gene Title	Gene Symbol
1558306_at	6,32899	0,0004658	thyroid adenoma associated	THADA
231857_s_at	6,08839	0,0024391	ATP/GTP binding protein-like 5	AGBL5
243774_at	5,57976	0,0025415	mucin 20, cell surface associated	MUC20
201367_s_at	4,22499	0,0012435	zinc finger protein 36, C3H type-like 2	ZFP36L2
237058_x_at	3,48199	0,0008399	solute carrier family 6	SLC6A13
1552696_at	3,37819	0,0053407	non imprinted in Prader-Willi	NIPA1
1555116_s_at	2,98021	0,0012541	solute carrier family 11	SLC11A1
1562695_at	2,83099	0,0009599	forkhead box N4	FOXN4
218741_at	2,55266	0,0102632	centromere protein M	CENPM
204344_s_at	2,51067	0,0008248	Sec23 homolog A (S. cerevisiae)	SEC23A
203163_at	2,40423	0,0036878	katanin p80	KATNB1
241782_at	2,23811	0,0085683	Nebulette	NEBL
1562028_at	1,99619	0,0063741	Cyclin D3	CCND3
218954_s_at	1,9924	0,0033594	BRF2, subunit of RNA polymerase III	BRF2
205839_s_at	1,97296	0,0020419	benzodiazapine receptor (peripheral)	BZRAP1
238600_at	1,97082	0,0096239	janus kinase and microtubule interacting protein 1	JAKMIP1
216191_s_at	1,92696	0,009489	T cell receptor alpha locus	TRA@
213006_at	1,89062	0,0074453	CCAAT/enhancer binding protein (C/EBP), delta	CEBPD
206653_at	1,85936	0,000666	polymerase (RNA) III (DNA directed) polypeptide G (32kD)	POLR3G
210719_s_at	1,80896	0,0021432	high-mobility group 20B	HMG20B
226072_at	1,80576	0,0023644	fucokinase	FUK
215407_s_at	1,77099	0,005554	astrotactin 2	ASTN2
224980_at	1,76462	0,0075903	LEM domain containing 2	LEMD2
220774_at	1,74988	0,0063365	dymeclin	DYM
212056_at	1,71318	0,0034272	KIAA0182	KIAA0182
214792_x_at	1,67666	0,0012805	vesicle-associated membrane protein 2	VAMP2
201313_at	1,66799	0,0008001	enolase 2 (gamma, neuronal)	ENO2
204156_at	1,66657	0,0039408	KIAA0999 protein	KIAA0999
238231_at	1,66594	0,0047534	Nuclear transcription factor Y, gamma	NFYC
215618_at	1,66265	0,0033644	Ras suppressor protein 1	RSU1
203469_s_at	1,64074	0,0039641	cyclin-dependent kinase 10	CDK10
206880_at	1,6264	0,0065823	purinergic receptor P2X, ligand-gated ion channel, 6	P2RX6
220221_at	1,6116	0,0089011	vacuolar protein sorting 13 homolog D (S. cerevisiae)	VPS13D
231003_at	1,60544	0,0051837	solute carrier family 35, member B3	SLC35B3
206748_s_at	1,60243	0,0034679	sperm associated antigen 9	SPAG9
1555843_at	1,6019	0,0001856	Heterogeneous nuclear ribonucleoprotein M	HNRNPM
210563_x_at	1,59919	0,0002258	CASP8 and FADD-like apoptosis regulator	CFLAR

1555259_at	1,57735	0,0095956	sterile alpha motif and leucine zipper containing kinase	ZAK
202518_at	1,57638	0,006959	B-cell CLL/lymphoma 7B	BCL7B
214196_s_at	1,56811	0,0077638	tripeptidyl peptidase I	TPP1
215269_at	1,56143	0,0052926	transmembrane protein 1	TMEM1
212521_s_at	1,54863	0,001385	phosphodiesterase 8A	PDE8A
232213_at	1,54697	0,0045362	Pellino homolog 1 (Drosophila)	PELI1
239233_at	1,54382	0,0050683	coiled-coil domain containing 88A	CCDC88A
1556467_at	1,53742	0,0079348	Zinc finger protein 80	ZNF80
1560145_at	1,53196	0,0098839	Muskelin 1	MKLN1
1555938_x_at	1,52895	0,0088522	vimentin	VIM
221838_at	1,52771	0,0057853	kelch-like 22 (Drosophila)	KLHL22
203264_s_at	1,50915	0,0029627	Cdc42 guanine nucleotide exchange factor (GEF) 9	ARHGEF9
209939_x_at	1,50678	0,0026933	CASP8 and FADD-like apoptosis regulator	CFLAR
244756_at	1,49987	0,0025035	ankyrin repeat domain 13 family, member D	ANKRD13D
1554086_at	1,49723	0,0004995	tubulin, gamma complex associated protein 3	TUBGCP3
225360_at	1,4766	0,005117	TraB domain containing	TRABD
1558116_x_at	1,45975	0,0047783	reversion-inducing-cysteine-rich protein with kazal motifs	RECK
228062_at	1,45753	0,0010916	nucleosome assembly protein 1-like 5	NAP1L5
206918_s_at	1,4509	0,0015095	copine I	CPNE1
228496_s_at	1,44968	0,0055578	Cysteine rich transmembrane BMP regulator 1 (chordin-like)	CRIM1
202740_at	1,4451	0,0037702	aminoacylase 1	ACY1
227878_s_at	1,44442	0,0013245	alkB, alkylation repair homolog 7 (E. coli)	ALKBH7
231959_at	1,44062	0,0017164	lin-52 homolog (C. elegans)	LIN52
215616_s_at	1,43184	0,0045848	jumonji domain containing 2B	JMJD2B
203994_s_at	1,40663	0,0074304	chromosome 21 open reading frame 2	C21orf2
219957_at	1,40429	0,0072414	RUN and FYVE domain containing 2	RUFY2
205213_at	1,39823	0,0104463	centaurin, beta 1	CENTB1
211370_s_at	1,39418	0,0012667	mitogen-activated protein kinase kinase 5	MAP2K5
207559_s_at	1,37511	0,0049674	zinc finger, MYM-type 3	ZMYM3
227064_at	1,375	0,0052124	ankyrin repeat domain 40	ANKRD40
230328_at	1,36563	0,0104824	RRN3 RNA polymerase I transcription factor homolog	LOC730092
201136_at	1,36352	0,0045015	proteolipid protein 2 (colonic epithelium-enriched)	PLP2
203674_at	1,35871	0,0075164	helicase with zinc finger	HELZ
244871_s_at	1,3556	0,0103846	ubiquitin specific peptidase 32	USP32
204902_s_at	1,34581	0,0070066	ATG4 autophagy related 4 homolog B (S. cerevisiae)	ATG4B
214918_at	1,33111	0,008819	heterogeneous nuclear ribonucleoprotein M	HNRNPM
225126_at	1,32862	0,0094217	mitochondrial ribosome recycling factor	MRRF
232030_at	1,32171	0,0102341	KIAA1632	KIAA1632
1569257_at	1,30466	0,0085017	formin-like 1	FMNL1
203514_at	1,30258	0,0080966	mitogen-activated protein kinase kinase kinase 3	MAP3K3

37424_at	1,29129	0,0040464	coiled-coil alpha-helical rod protein 1	CCHCR1
1557804_at	1,27916	0,0091668	Cathepsin B mRNA, 5' UTR variant	---
233841_s_at	1,26386	0,0082203	suppressor of defective silencing 3 homolog (S. cerevisiae)	SUDS3
223430_at	1,25933	0,0066801	SNF1-like kinase 2	SNF1LK2
242243_at	1,24393	0,0090739	TATA element modulatory factor 1	TMF1
223647_x_at	1,22898	0,0064339	HscB iron-sulfur cluster co-chaperone homolog (E. coli)	HSCB
229980_s_at	1,22493	0,0019934	sorting nexin 5	SNX5
1552329_at	1,19364	0,0022315	retinoblastoma binding protein 6	RBBP6
225910_at	1,16709	0,0074809	helicase with zinc finger	HELZ
218478_s_at	1,15189	0,0094328	zinc finger, CCHC domain containing 8	ZCCHC8
209472_at	1,12188	0,0090171	cysteine conjugate-beta lyase 2	CCBL2
222388_s_at	-1,1432	0,0090193	vacuolar protein sorting 35 homolog (S. cerevisiae)	VPS35
202522_at	-1,1907	0,0081302	phosphatidylinositol transfer protein, beta	PITPNB
226320_at	-1,2	0,0099819	THO complex 4	THOC4
1554149_at	-1,2252	0,0070013	claudin domain containing 1	CLDND1
226916_x_at	-1,2498	0,0049585	dipeptidyl-peptidase 9	DPP9
203880_at	-1,2633	0,0057562	COX17 cytochrome c oxidase assembly homolog (S. cerevisiae)	COX17
212632_at	-1,2776	0,0067539	syntaxin 7	STX7
213223_at	-1,3022	0,0065303	ribosomal protein L28	RPL28
218955_at	-1,3289	0,0038134	BRF2, subunit of RNA polymerase III transcription initiation factor	BRF2
220315_at	-1,3587	0,0091039	poly (ADP-ribose) polymerase family, member 11	PARP11
207057_at	-1,3819	0,0079308	solute carrier family 16, member 7	SLC16A7
228093_at	-1,3896	0,0008657	zinc finger protein 599	ZNF599
205084_at	-1,3968	0,0064425	B-cell receptor-associated protein 29	BCAP29
205661_s_at	-1,4138	0,0008906	FAD1 flavin adenine dinucleotide synthetase homolog	FLAD1
204191_at	-1,4335	0,0071291	interferon (alpha, beta and omega) receptor 1	IFNAR1
208814_at	-1,4342	0,0030714	Heat shock 70kDa protein 4	HSPA4
226504_at	-1,4849	0,0029322	family with sequence similarity 109, member B	FAM109B
204173_at	-1,5228	0,009641	myosin, light chain 6B, alkali, smooth muscle and non-muscle	MYL6B
218966_at	-1,526	0,0038248	myosin VC	MYO5C
210115_at	-1,5487	0,0025829	ribosomal protein L39-like	RPL39L
210672_s_at	-1,6063	0,0092833	chromosome 16 open reading frame 35	C16orf35
225715_at	-1,6101	0,0043118	raptor	KIAA1303
234921_at	-1,6212	0,0050798	zinc finger protein 470	ZNF470
238547_at	-1,632	0,0094537	hexamthylene bis-acetamide inducible 2	HEXIM2
228958_at	-1,6373	0,004992	zinc finger protein 19	ZNF19
220032_at	-1,6541	0,0090616	chromosome 7 open reading frame 58	C7orf58
222520_s_at	-1,7009	0,0089253	intraflagellar transport 57 homolog (Chlamydomonas)	IFT57
226770_at	-1,7558	0,0038036	membrane associated guanylate kinase	MAGI3
212002_at	-1,8064	0,0018944	chromosome 1 open reading frame 144	C1orf144

1554586_a_at	-1,8528	0,0051946	Rho-related BTB domain containing 2	RHOBTB2
1554616_at	-1,856	0,0008605	serpin peptidase inhibitor, clade B (ovalbumin), member 8	SERPINB8
229365_at	-1,9253	0,0006716	protein phosphatase 1, regulatory (inhibitor) subunit 3F	PPP1R3F
241133_at	-1,9765	0,0079569	T-cell receptor beta (TCRB) mRNA (HLA-A3, 29; B7, 44; DR2, 7)	---
1555284_at	-2,0549	0,0016568	amyotrophic lateral sclerosis 2 (juvenile)	ALS2
221057_at	-2,0753	0,006812	spermatogenesis associated 1	SPATA1
223828_s_at	-2,0997	0,0071572	lectin, galactoside-binding, soluble, 12 (galectin 12)	LGALS12
239369_at	-2,1717	0,002087	lipocalin 8	LCN8
242178_at	-2,2545	0,0042791	lipase, member I	LIPI
206794_at	-2,2598	0,0050741	v-erb-a erythroblastic leukemia viral oncogene homolog 4	ERBB4
204517_at	-2,2729	0,0049548	peptidylprolyl isomerase C (cyclophilin C)	PPIC
220463_at	-2,4355	0,0056032	transient receptor potential cation channel, subfamily M	TRPM3
1558722_at	-2,4575	0,003588	zinc finger protein 252	ZNF252
207466_at	-2,5137	0,0010555	galanin prepropeptide	GAL
212226_s_at	-2,6872	0,0058139	phosphatidic acid phosphatase type 2B	PPAP2B
204472_at	-2,8931	0,0013734	GTP binding protein overexpressed in skeletal muscle	GEM
204932_at	-2,9164	0,0071887	tumor necrosis factor receptor superfamily, member 11b	TNFRSF11B
210637_at	-3,1937	0,0080335	tachykinin receptor 1	TACR1
230015_at	-4,1687	0,0022496	progressive rod-cone degeneration	PRCD
1557136_at	-4,3392	0,0033758	ATPase type 13A4	ATP13A4

FC = Fold change

Table 3. Gene expression signature in PBMCs of the pairwise comparison (Group 1 *versus* Group 3)

Probe Set ID	FC	p.value	Gene Title	Gene Symbol
213831_at	162,5052	0,004604	major histocompatibility complex, class II, DQ alpha 1	HLA-DQA1
209480_at	131,3122	0,005414	major histocompatibility complex, class II, DQ beta 1	HLA-DQB1
213660_s_at	3,122353	0,006437	topoisomerase (DNA) III beta	TOP3B
219596_at	2,818932	0,005966	THAP domain containing 10	THAP10
208587_s_at	2,70578	0,003631	olfactory receptor, family 1, subfamily E, member 1	OR1E1
211654_x_at	2,538185	0,006057	major histocompatibility complex, class II, DQ beta 1	HLA-DQB1
1568665_at	2,249213	0,002677	ring finger protein 103	RNF103
220058_at	2,090948	0,00085	chromosome 17 open reading frame 39	C17orf39
220135_s_at	1,945639	0,007855	solute carrier family 7	SLC7A9
1562005_at	1,819878	0,005522	Ras and Rab interactor 3	RIN3
210486_at	1,804503	0,006544	ankyrin repeat and MYND domain containing 1	ANKMY1
211571_s_at	1,728594	0,009241	versican	VCAN
204663_at	1,447246	0,00551	malic enzyme 3, NADP(+)-dependent, mitochondrial	ME3
231824_at	1,420775	0,001005	La ribonucleoprotein domain family, member 2	LARP2
210563_x_at	1,408201	0,004487	CASP8 and FADD-like apoptosis regulator	CFLAR
224969_at	1,397396	0,002291	ataxin 7-like 3	ATXN7L3
204423_at	1,371274	0,004728	muskelin 1	MKLN1
216640_s_at	1,371206	0,006086	protein disulfide isomerase family A, member 6	PDIA6
1552719_at	1,368399	0,005884	cancer susceptibility candidate 4	CASC4
224627_at	1,367685	0,002806	glucosidase, beta (bile acid) 2	GBA2
244756_at	1,35715	0,007809	ankyrin repeat domain 13 family, member D	ANKRD13D
232676_x_at	1,326846	0,005346	myelin expression factor 2	MYEF2
214960_at	1,319963	0,0093	apoptosis inhibitor 5	API5
217933_s_at	1,319662	0,001837	leucine aminopeptidase 3	LAP3
232809_s_at	1,319578	0,010189	Fms-related tyrosine kinase 1	FLT1
223393_s_at	1,318151	0,008821	teashirt zinc finger homeobox 3	TSHZ3
221542_s_at	1,303387	0,007663	ER lipid raft associated 2	ERLIN2
1554365_a_at	1,287141	0,006406	protein phosphatase 2	PPP2R5C
217872_at	1,279198	0,00202	PIH1 domain containing 1	PIH1D1
222978_at	1,269993	0,0027	surfeit 4	SURF4
202433_at	1,240882	0,008987	solute carrier family 35, member B1	SLC35B1
238538_at	1,23786	0,00116	ankyrin repeat domain 11	ANKRD11
235314_at	1,234111	0,005393	ribosomal protein L32 pseudogene 3	RPL32P3
204342_at	1,222452	0,007115	solute carrier family 25	SLC25A24
217795_s_at	1,219633	0,006066	transmembrane protein 43	TMEM43
204799_at	1,211079	0,007909	zinc finger, BED-type containing 4	ZBED4
202375_at	1,208208	0,00338	SEC24 related gene family, member D (S. cerevisiae)	SEC24D
225075_at	1,206231	0,004929	p53 and DNA damage regulated 1	PDRG1

218827_s_at	1,203032	0,007135	centrosomal protein 192kDa	CEP192
227521_at	1,196263	0,00591	F-box protein 33	FBXO33
232652_x_at	1,193843	0,009985	SCAN domain containing 1	SCAND1
218852_at	1,186898	0,007962	protein phosphatase 2 (formerly 2A)	PPP2R3C
226409_at	1,18685	0,006633	TBC1 domain family, member 20	TBC1D20
205633_s_at	1,186211	0,004801	aminolevulinate, delta-, synthase 1	ALAS1
209103_s_at	1,184846	0,010069	ubiquitin fusion degradation 1 like (yeast)	UFD1L
201964_at	1,158858	1,99E-05	senataxin	SETX
225049_at	-1,13591	0,008979	biogenesis of lysosome-related organelles complex-1	BLOC1S2
212539_at	-1,1362	0,002427	chromodomain helicase DNA binding protein 1-like	CHD1L
209932_s_at	-1,14473	0,003271	deoxyuridine triphosphatase	DUT
203171_s_at	-1,16883	0,006634	KIAA0409	KIAA0409
223089_at	-1,20058	0,003734	vezatin, adherens junctions transmembrane protein	VEZT
227203_at	-1,20465	0,003334	F-box and leucine-rich repeat protein 17	FBXL17
219041_s_at	-1,21447	0,006104	replication initiator 1	REPIN1
201661_s_at	-1,21479	0,007739	acyl-CoA synthetase long-chain family member 3	ACSL3
215983_s_at	-1,22629	0,002167	UBX domain containing 6	UBXD6
202867_s_at	-1,23524	0,003463	DnaJ (Hsp40) homolog, subfamily B, member 12	DNAJB12
225581_s_at	-1,25401	0,005568	mitochondrial ribosomal protein L50	MRPL50
203708_at	-1,27778	0,003882	phosphodiesterase 4B, cAMP-specific	PDE4B
228992_at	-1,28352	0,009711	Mediator complex subunit 28	MED28
205672_at	-1,2892	0,00351	xeroderma pigmentosum, complementation group A	XPA
229491_at	-1,31798	0,008838	Na+/H+ exchanger domain containing 2	NHEDC2
203196_at	-1,33722	0,010359	ATP-binding cassette	ABCC4
218808_at	-1,34005	0,010296	DALR anticodon binding domain containing 3	DALRD3
201163_s_at	-1,36418	0,005093	insulin-like growth factor binding protein 7	IGFBP7
220936_s_at	-1,36947	0,005027	H2A histone family, member J	H2AFJ
235074_at	-1,3772	0,008085	sprouty-related, EVH1 domain containing 1	SPRED1
219805_at	-1,37761	0,010478	chromosome X open reading frame 56	CXorf56
222744_s_at	-1,37926	0,002013	trimethyllysine hydroxylase, epsilon	TMLHE
229138_at	-1,38052	0,010106	poly (ADP-ribose) polymerase family, member 11	PARP11
65718_at	-1,38203	0,002905	G protein-coupled receptor 124	GPR124
239391_at	-1,39483	0,008418	Family with sequence similarity 120A opposite strand	FAM120AOS
213952_s_at	-1,39584	0,009103	Arachidonate 5-lipoxygenase	ALOX5
227001_at	-1,41026	0,008309	NIPA-like domain containing 2	NPAL2
1563090_at	-1,4239	0,001308	coiled-coil domain containing 33	CCDC33
216583_x_at	-1,42433	0,00556	nucleolar protein family A, member 2	NOLA2
228958_at	-1,42704	0,006512	zinc finger protein 19	ZNF19
1561965_at	-1,42913	0,009344	Small nuclear ribonucleoprotein polypeptide B"	SNRPB2
203858_s_at	-1,44262	0,008655	COX10 homolog, cytochrome c oxidase assembly protein	COX10

227386_s_at	-1,45064	0,009883	transmembrane protein 200B	TMEM200B
212385_at	-1,45953	0,009025	transcription factor 4	TCF4
238660_at	-1,47485	0,001054	WD repeat and FYVE domain containing 3	WDFY3
233065_at	-1,48068	0,009467	ring finger protein 207	RNF207
214213_x_at	-1,48785	0,006552	Lamin A/C	LMNA
1570342_at	-1,50337	0,007252	natural killer-tumor recognition sequence	NKTR
1561190_at	-1,53872	0,007972	cyclin-dependent kinase-like 3	CDKL3
227421_at	-1,55346	0,006082	chromosome 21 open reading frame 57	C21orf57
1553134_s_at	-1,56171	0,005744	chromosome 9 open reading frame 72	C9orf72
220973_s_at	-1,56628	0,000832	SHANK-associated RH domain interactor	SHARPIN
1569815_x_at	-1,57233	0,006381	striatin, calmodulin binding protein	STRN
1555868_at	-1,59465	0,002235	Similar to hCG2030186	LOC729070
1557480_a_at	-1,59962	0,006417	dysferlin interacting protein 1	DYSFIP1
210612_s_at	-1,64353	0,000182	synaptojanin 2	SYNJ2
1568763_s_at	-1,67309	0,007987	programmed cell death 6	LOC728613 / PDCD6
200770_s_at	-1,68544	0,003566	laminin, gamma 1 (formerly LAMB2)	LAMC1
1557047_at	-1,68637	0,004565	YEATS domain containing 2	YEATS2
210791_s_at	-1,70591	0,006051	Rho GTPase-activating protein	RICS
206010_at	-1,71335	0,005973	hyaluronan binding protein 2	HABP2
230896_at	-1,71737	0,007511	coiled-coil domain containing 4	CCDC4
1553674_at	-1,73074	0,006342	leucine rich repeat containing 44	LRRC44
241599_at	-1,74926	0,004763	LSM11, U7 small nuclear RNA associated	LSM11
210290_at	-1,75513	0,008438	zinc finger protein 174	ZNF174
217484_at	-1,83408	0,001916	complement component (3b/4b) receptor 1	CR1
206134_at	-1,85225	0,005217	ADAM-like, decysin 1	ADAMDEC1
221610_s_at	-1,86096	0,004489	signal transducing adaptor family member 2	STAP2
202283_at	-1,86159	0,000684	serpin peptidase inhibitor, clade F	SERPINF1
56919_at	-1,86322	0,00277	WD repeat domain 48	WDR48
223137_at	-1,87367	0,007377	zinc finger, DHHC-type containing 4	ZDHHC4
210313_at	-1,89086	0,006878	leukocyte immunoglobulin-like receptor, subfamily A	LILRA4
214516_at	-1,9206	0,010053	histone cluster 1, H4i	HIST1H4A
216821_at	-1,93697	0,002337	keratin 8 /// similar to keratin 8	KRT8
233015_at	-1,94867	0,008364	muscleblind-like (Drosophila)	MBNL1
1564301_a_at	-1,95886	0,005799	RPA interacting protein	RPAIN
202620_s_at	-1,99905	0,007319	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	PLOD2
1558557_at	-2,0348	0,006659	chromosome 16 open reading frame 62	C16orf62
216077_s_at	-2,0858	0,008211	l(3)mbt-like (Drosophila)	L3MBTL
239816_at	-2,09372	0,009613	Polymerase (DNA-directed), delta 3, accessory subunit	POLD3
206726_at	-2,09808	0,003958	prostaglandin D2 synthase, hematopoietic	PGDS
224170_s_at	-2,10913	0,00271	tubby like protein 4	TULP4

225654_at	-2,12891	0,002151	nuclear receptor binding SET domain protein 1	NSD1
229883_at	-2,16308	0,008253	glutamate receptor, ionotropic	GRIN2D
32540_at	-2,17771	0,005252	Protein phosphatase 3 (formerly 2B), catalytic subunit	PPP3CC
203461_at	-2,18344	0,004255	chromodomain helicase DNA binding protein 2	CHD2
239617_at	-2,3457	0,007495	SEC13 homolog (S. cerevisiae)	SEC13
1559561_at	-2,43164	0,007844	F-box protein, helicase, 18	FBXO18
206353_at	-2,4636	0,009116	cytochrome c oxidase subunit VIa polypeptide 2	COX6A2
1553417_at	-2,57807	0,000833	chromosome 11 open reading frame 44	C11orf44
215765_at	-2,77576	0,007107	leucine rich repeat containing 41	LRRC41
1569110_x_at	-2,82335	0,001456	programmed cell death 6 pseudogene	LOC728613
220574_at	-2,98725	0,006667	sema domain, transmembrane domain ™	SEMA6D
1559393_at	-3,28934	0,002776	aldehyde dehydrogenase 1 family, member L2	ALDH1L2
218625_at	-3,62643	0,004086	neuritin 1	NRN1
212142_at	-4,46456	0,000197	minichromosome maintenance complex component 4	MCM4
1556099_at	-6,15817	0,010093	hCG2025798	hCG_2025798

FC = Fold change

Table 4. Gene expression signature in PBMCs of the pairwise comparison (Group 2 *versus* Group 3)

Probe Set ID	FC	p.value	Gene Title	Gene Symbol
211011_at	4,11766	0,00048	collagen, type XIX, alpha 1	COL19A1
1559507_at	2,36748	0,00359	similar to hCG2038897	LOC100130357
204517_at	2,21678	0,00859	peptidylprolyl isomerase C (cyclophilin C)	PPIC
1554586_a_at	2,13082	0,00752	Rho-related BTB domain containing 2	RHOBTB2
219866_at	2,03557	0,00391	chloride intracellular channel 5	CLIC5
230484_at	2,02355	0,00303	Choline dehydrogenase	CHDH
1559921_at	1,86246	0,00398	platelet/endothelial cell adhesion molecule (CD31 antigen)	PECAM1
225186_at	1,84576	0,00513	Ras association (RalGDS/AF-6)	RAPH1
227230_s_at	1,81978	0,00729	KIAA1211 protein	KIAA1211
238925_at	1,76352	0,00647	syntrophin, beta 2	SNTB2
235643_at	1,75651	0,00354	sterile alpha motif domain containing 9-like	SAMD9L
218663_at	1,73293	0,00438	non-SMC condensin I complex, subunit G	NCAPG
208129_x_at	1,7269	0,00152	runt-related transcription factor 1	RUNX1
202344_at	1,58812	0,00761	heat shock transcription factor 1	HSF1
1553612_at	1,48325	0,001	zinc finger protein 354B	ZNF354B
206857_s_at	1,47918	0,00697	FK506 binding protein 1B, 12.6 kDa	FKBP1B
240486_at	1,4762	0,01014	Helicase with zinc finger	HELZ
222499_at	1,42983	0,00383	mitochondrial ribosomal protein S16	MRPS16
209414_at	1,42481	0,00296	fizzy/cell division cycle 20 related 1 (Drosophila)	FZR1
203432_at	1,40549	0,00988	thymopoietin	TMPO
220315_at	1,39949	0,00865	poly (ADP-ribose) polymerase family, member 11	PARP11
204423_at	1,34394	0,00698	muskelin 1, intracellular mediator containing kelch motifs	MKLN1
218589_at	1,31706	0,00251	purinergic receptor P2Y, G-protein coupled, 5	P2RY5
226504_at	1,28911	0,00647	family with sequence similarity 109, member B	FAM109B
204544_at	1,24155	0,0001	Hermansky-Pudlak syndrome 5	HPS5
208840_s_at	1,23651	0,00411	GTPase activating protein (SH3 domain) binding protein 2	G3BP2
203880_at	1,23294	0,00419	COX17 cytochrome c oxidase assembly homolog (S. cerevisiae)	COX17
210218_s_at	1,2282	0,00483	SP100 nuclear antigen	SP100
209103_s_at	1,21613	0,00364	ubiquitin fusion degradation 1 like (yeast)	UFD1L
205633_s_at	1,21484	0,00414	aminolevulinate, delta-, synthase 1	ALAS1
221277_s_at	1,18749	0,0097	pseudouridylate synthase 3	PUS3
37577_at	1,15949	0,00456	Rho GTPase activating protein 19	ARHGAP19
217834_s_at	1,15647	0,00636	synaptotagmin binding, cytoplasmic RNA interacting protein	SYNCRIP
201585_s_at	1,1454	0,00138	splicing factor proline/glutamine-rich	SFPQ
227521_at	1,13748	0,00213	F-box protein 33	FBXO33
201086_x_at	1,09188	0,00028	SON DNA binding protein	SON
205105_at	1,06989	0,00281	mannosidase, alpha, class 2A, member 1	MAN2A1
201032_at	-1,1094	0,00692	bladder cancer associated protein	BLCAP

203594_at	-1,1132	0,00616	RNA terminal phosphate cyclase domain 1	RTCD1
232946_s_at	-1,1326	0,00666	NAD synthetase 1	NADSYN1
209472_at	-1,1353	0,00203	cysteine conjugate-beta lyase 2	CCBL2
214276_at	-1,1422	0,00376	Kruppel-like factor 12	KLF12
226318_at	-1,166	0,00441	transforming growth factor beta regulator 1	TBRG1
223146_at	-1,1911	0,00726	WD repeat domain 33	WDR33
228622_s_at	-1,1989	0,00693	DnaJ (Hsp40) homolog, subfamily C, member 4	DNAJC4
225559_at	-1,2064	0,00785	chromosome 3 open reading frame 19	C3orf19
213161_at	-1,2259	0,01046	chromosome 9 open reading frame 97	C9orf97
31807_at	-1,2525	0,00602	DEAD (Asp-Glu-Ala-Asp) box polypeptide 49	DDX49
229897_at	-1,2558	0,0062	Zinc finger protein 641	ZNF641
221742_at	-1,2713	0,00941	CUG triplet repeat, RNA binding protein 1	CUGBP1
236814_at	-1,2725	0,00951	Mdm4 p53 binding protein homolog (mouse)	MDM4
205282_at	-1,2775	0,00761	low density lipoprotein receptor-related protein 8	LRP8
222149_x_at	-1,2827	0,01043	golgi autoantigen, golgin subfamily a, 8A	FLJ32679
214663_at	-1,2839	0,00281	receptor interacting protein kinase 5	RIPK5
225002_s_at	-1,3085	0,00704	sulfatase modifying factor 2	SUMF2
201219_at	-1,3117	0,00601	C-terminal binding protein 2	CTBP2
215399_s_at	-1,3194	0,00524	amplified in osteosarcoma	OS9
229491_at	-1,3251	0,00559	Na+/H+ exchanger domain containing 2	NHEDC2
202098_s_at	-1,3494	0,01006	protein arginine methyltransferase 2	PRMT2
214144_at	-1,3535	0,00853	Polymerase (RNA) II (DNA directed) polypeptide D	POLR2D
208684_at	-1,3714	0,00563	coatomer protein complex, subunit alpha	COPA
218784_s_at	-1,3718	0,00629	chromosome 6 open reading frame 64	C6orf64
229035_s_at	-1,3798	0,00881	kelch domain containing 4	KLHDC4
225126_at	-1,3896	0,00228	mitochondrial ribosome recycling factor	MRRF
218437_s_at	-1,3908	0,00259	leucine zipper transcription factor-like 1	LZTFL1
32402_s_at	-1,3972	0,00899	symplekin	SYMPK
232486_at	-1,4199	0,00847	leucine rich repeat and fibronectin type III domain containing 1	LRFN1
230707_at	-1,429	0,00855	sortilin-related receptor, L(DLR class) A repeats-containing	SORL1
232912_at	-1,434	0,00365	G protein-coupled receptor 180	GPR180
235333_at	-1,4419	0,00129	UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase	B4GALT6
1561965_at	-1,4506	0,0087	Small nuclear ribonucleoprotein polypeptide B"	SNRPB2
232213_at	-1,4567	0,00997	Pellino homolog 1 (Drosophila)	PELI1
214915_at	-1,475	0,00979	Zinc finger protein 362	ZNF362
231958_at	-1,479	0,00045	Chromosome 3 open reading frame 31	C3orf31
220774_at	-1,4846	0,00361	dymeclin	DYM
232304_at	-1,495	0,01	Pellino homolog 1 (Drosophila)	PELI1
229693_at	-1,5131	0,00848	RIKEN cDNA A730055C05-like	LOC388335
210290_at	-1,5254	0,00763	zinc finger protein 174	ZNF174

1562434_at	-1,585	0,00013	unkempt homolog (Drosophila)	UNK
214792_x_at	-1,5964	0,00023	vesicle-associated membrane protein 2 (synaptobrevin 2)	VAMP2
239163_at	-1,6097	0,00873	ubiquitin-conjugating enzyme E2B (RAD6 homolog)	UBE2B
212056_at	-1,6097	0,00964	KIAA0182	KIAA0182
243294_at	-1,6169	0,0088	zinc finger protein 780B	ZNF780B
1555259_at	-1,619	0,00525	sterile alpha motif and leucine zipper containing kinase AZK	ZAK
235532_at	-1,6583	0,00522	phosphatidylinositol glycan anchor biosynthesis, class M	PIGM
229883_at	-1,6792	0,00685	glutamate receptor, ionotropic, N-methyl D-aspartate 2D	GRIN2D
206838_at	-1,6889	0,00894	T-box 19	TBX19
224816_at	-1,706	0,0089	Chromosome 7 open reading frame 20	C7orf20
1559479_at	-1,7588	0,00464	Phosphatidylinositol 4-kinase type 2 beta	PI4K2B
239493_at	-1,7752	0,00789	ribosomal protein L7	hCG_2015956
1558217_at	-1,7775	0,00135	schlafen family member 13	SLFN13
224027_at	-1,8551	0,00634	chemokine (C-C motif) ligand 28	CCL28
215645_at	-1,8595	0,00515	folliculin	FLCN
215243_s_at	-1,9657	0,00981	gap junction protein, beta 3, 31kDa	GJB3
212853_at	-2,1001	0,00941	DCN1, defective in cullin neddylation 1, domain containing 4	DCUN1D4
229037_at	-2,1124	0,00479	Tripartite motif-containing 69	TRIM69
204485_s_at	-2,1401	0,00942	target of myb1 (chicken)-like 1	TOM1L1
209160_at	-2,1972	0,00302	aldo-keto reductase family 1, member C3	AKR1C3
216821_at	-2,2483	0,00087	keratin 8 /// similar to keratin 8 /// keratin 8 pseudogene 9	KRT8
1560499_at	-2,2565	0,00548	chromosome 14 open reading frame 64	C14orf64
238613_at	-2,2807	0,00368	sterile alpha motif and leucine zipper containing kinase AZK	ZAK
206726_at	-2,3291	0,00937	prostaglandin D2 synthase, hematopoietic	PGDS
204542_at	-2,3703	0,00675	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)	ST6GALNAC2
1558053_s_at	-2,5199	0,01	transmembrane emp24 protein transport domain containing 4	TMED4
215510_at	-2,9158	0,00298	ets variant gene 2	ETV2
1561477_at	-2,9969	0,00753	coiled-coil domain containing 144A	CCDC144A
216882_s_at	-3,1311	0,00378	nebulette	NEBL
226303_at	-3,1651	0,01016	phosphoglucomutase 5	PGM5
222722_at	-3,376	0,0103	osteoglycin	OGN
217753_s_at	-3,514	3,36E-07	ribosomal protein S26 /// similar to 40S ribosomal protein S26	RPS26
234936_s_at	-3,5684	0,00161	coiled-coil and C2 domain containing 2A	CC2D2A
241631_at	-3,6965	0,00462	Full length insert cDNA clone YI72E07	---
215723_s_at	-3,7794	0,00588	phospholipase D1, phosphatidylcholine-specific	PLD1
201367_s_at	-3,9706	0,00707	zinc finger protein 36, C3H type-like 2	ZFP36L2
237058_x_at	-4,0026	0,0032	solute carrier family 6	SLC6A13
217014_s_at	-4,0888	0,00079	alpha-2-glycoprotein 1, zinc-binding	AZGP1
220842_at	-4,1915	0,00963	Abelson helper integration site 1	AHI1
243804_at	-4,6216	0,0046	myotubularin related protein 7	MTMR7

222381_at	-4,7161	0,00257	Aryl-hydrocarbon receptor repressor	PDCD6
1566087_at	-4,9962	0,00821	MRNA; cDNA DKFZp667A1714 (from clone DKFZp667A1714)	---
218186_at	-7,5836	0,00844	RAB25, member RAS oncogene family	RAB25

FC = Fold change

Table 5- Probe set ID of the gene expression in PBMCs cells of the sample and its commercial codes

Groups/Probe Set ID	Gene Title	Gene Symbol	Commercial Code*
(Group 1 + 2) *vs* Group 3			
213831_at	Major Histocompatibility Complex, class II, DQ alpha 1	*HLA-DQA1*	Hs03007426-mH
212777_at	Son of sevenless homolog 1 (Drosophila)	*SOS1*	Hs00893134_m1
206726_at	Prostaglandin D2 synthase, hematopoietic	*HPGDS*	Hs01023933_m1
222381_at	Aryl-hidrocarbon receptor repressor	*PDCD6*	Hs00918237_m1
Group 1 *vs* Group 2			
231857_s_at	ATP/GTP binding protein-like 5	*AGBL5*	Hs01005454_g1
216191_s_at	T-cell receptor alpha locus	*TRDV3*	Hs00612238_m1
212226_s_at	Phosphatidic acid phosphatase type 2B	*PPAP2B*	Hs00170359_m1
223828_s_at	Lectin, Galactoside-binding, soluble, 12 (galectin 12)	*LGALS12*	Hs00263821_m1
Group 1 *vs* Group 3			
209480_at	Major Histocompatibility Complex, class II, DQ beta 1	*HLA-DQB1*	Hs03054971_m1
1562005_at	Ras and Rab interaction 3	*RIN3*	Hs00227365_m1
211571_s_at	Versican	*VCAN*	Hs00171642_m1
239617_at	SEC13 homolog (S. cerevisiae)	*SEC13*	Hs01115007_m1
212142_at	Minichromosome maintenance	*MCM4*	Hs00907398_m1
Group 2 *vs* Group 3			
204517_at	Peptidylprolyl isomerase C (cyclosphilin C)	*PPIC*	Hs00917412_m1
1554586_a_at	Rho-related BTB domain containing 2	*RHOBTB2*	Hs01598095_g1

1559921_at	Platelet/endotelial cell adhesion molecule (CD31 antigen)	*PECAM1*	Hs00169777_m1
224027_at	Chemikine (cc-motif) ligand 28	*CCL28*	Hs_00955110_m1
237058_x_at	Solute carrier Family 6 (neurotransmitter transporter, GABA), member 13	*SLC6A13*	Hs00213290_m1

FC – Fold Change; * Commercial Code by Applied Biosystems

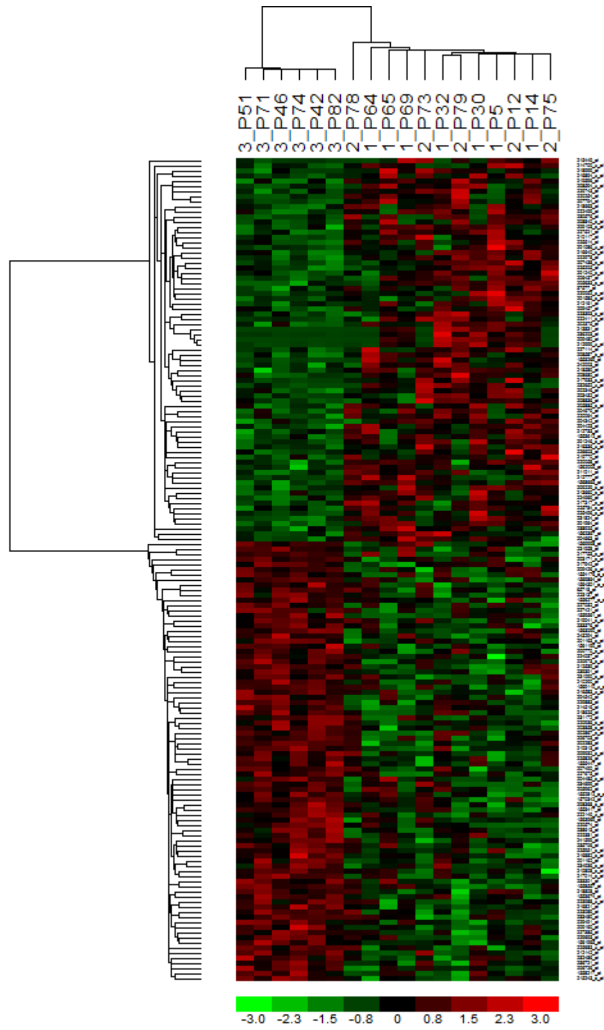

Figure 1. Hierarchical clustering of genes with significantly different expression changes between Groups 1 + 2 *versus* Group 3. Red and green indicate postevent up- and downregulation, respectively, and intensity of color indicates the degree of gene regulation shown on the scale below.

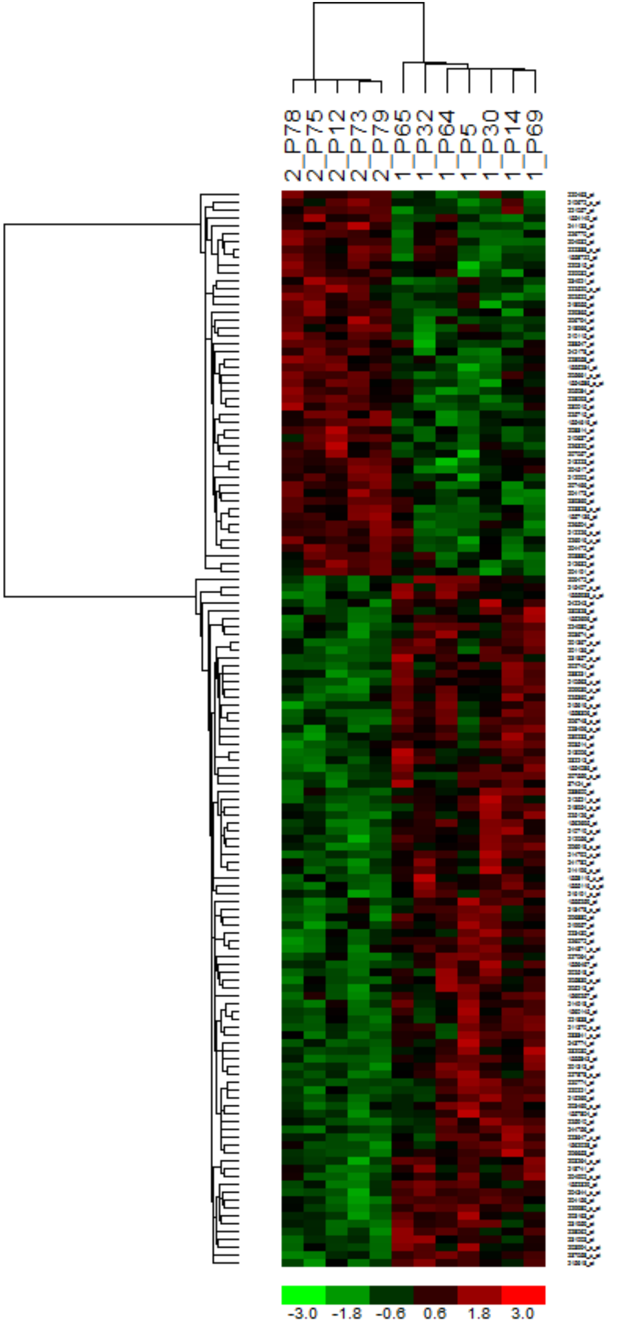

Figure 2. Hierarchical clustering of genes with significantly different expression changes between Group 1 *versus* Group 2. Red and green indicate postevent up- and downregulation, respectively, and intensity of color indicates the degree of gene regulation shown on the scale below.

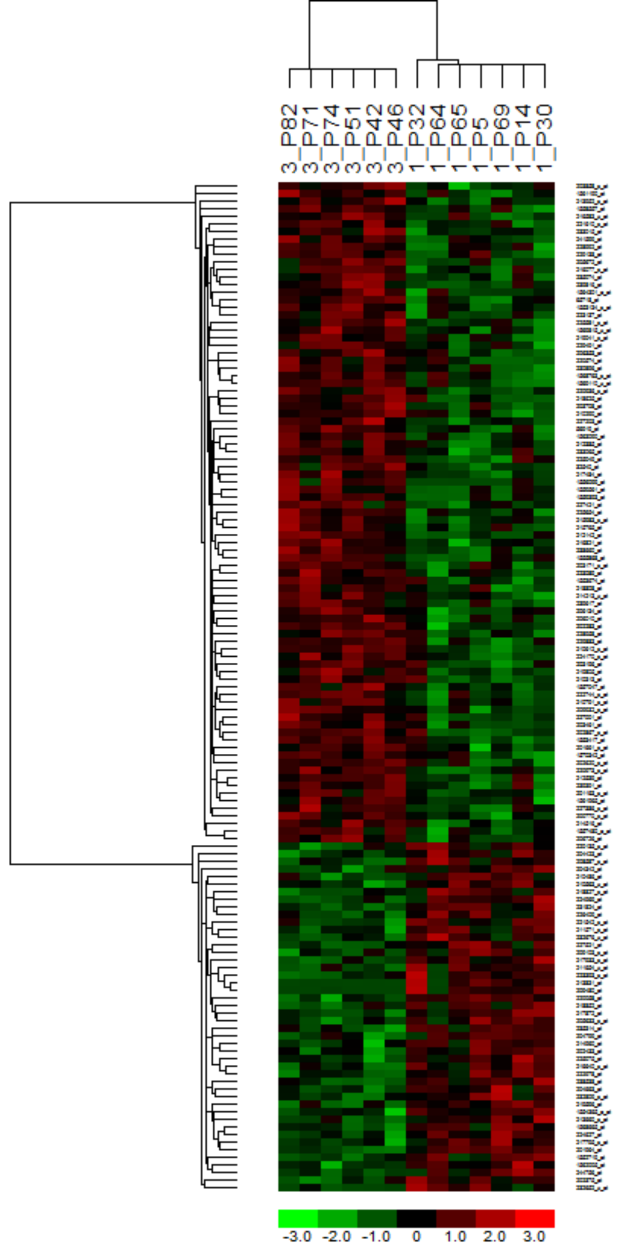

Figure 3. Hierarchical clustering of genes with significantly different expression changes between Group 1 *versus* Group 3. Red and green indicate postevent up- and downregulation, respectively, and intensity of color indicates the degree of gene regulation shown on the scale below.

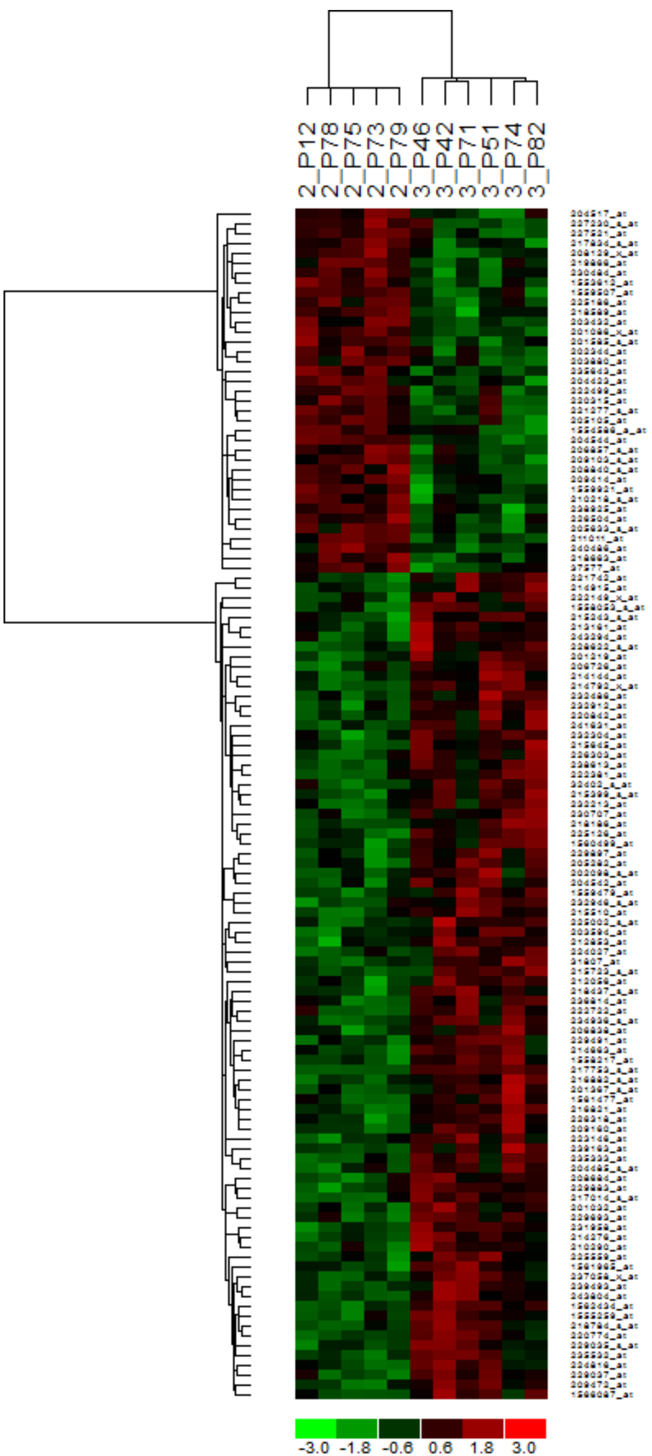

Figure 4. Hierarchical clustering of genes with significantly different expression changes between Group 2 *versus* Group 3. Red and green indicate postevent up- and downregulation, respectively, and intensity of color indicates the degree of gene regulation shown on the scale below.

4 DISCUSSÃO

Este presente estudo teve por objetivo investigar a frequência de lesões irreversíveis no DNA, por meio do teste do Micronúcleo, assim como avaliar pela técnica de *microarray* a expressão gênica de indivíduos portadores de DM2 (compensado e não compensado metabolicamente), dislipidemia e/ou periodontite crônica. Pelo nosso conhecimento, este é o primeiro estudo na literatura científica que se propôs a pesquisar a associação de danos no DNA e a prospectar o perfil da expressão gênica, em indivíduos que apresentam simultaneamente (ou não) essas três patologias.

Os resultados do teste do micronúcleo aqui apresentados indicaram uma associação entre o DM2 e danos irreversíveis no DNA, considerando a frequência de células binucleadas com micronúcleo (MCF) e a frequência de micronúcleos (MNF), especialmente levando-se em conta os resultados observados para o grupo 2 (diabetes compensado metabolicamente com dislipidemia e periodontite). Avaliando o teste do Micronúcleo e o DM, foi encontrado na literatura, que Andreassi et al. [3] (2005) mostraram que o DM2 foi o principal determinante no aumento da frequência de MN em linfócitos circulantes de pacientes com doença cardíaca isquêmica. O aumento da frequência de MN também foi observado por Martínez-Perez et al. [70] (2007) em pacientes com DM2 que não tinham complicações micro ou macrovasculares. Zúñiga-González et al. [127] (2007), investigando pacientes diabéticos do tipo 1, observaram um aumento definitivo da frequência de MN em circunstância de indivíduos diabéticos não compensados (HbA1C > 7%). Além disso, também encontraram uma redução significativa de MNs após suplementação de ácido fólico por 30 dias. Diferentemente, Cinkilic et al. [17] (2009) não conseguiram encontrar qualquer diferença significativa

entre a freqüência de MN em pacientes diabéticos do tipo 1, em comparação com o grupo controle.

Inesperadamente, o presente estudo mostrou níveis mais elevados da frequência de MN em pacientes com DM2 compensado (grupo 2) do que em pacientes com DM2 não compensado metabolicamente (grupo 1). Este resultado pode ser explicado pelo fato de os pacientes do grupo 2 apresentarem altos níveis de Proteína C-reativa, triglicerídeos, IMC e circunferência abdominal em relação aos outros grupos estudados. Sabe-se que a Proteína C-reativa é um marcador bem estabelecido de inflamação e doença cardiovascular [92] e que pode ser reconhecida como um parâmetro de estresse oxidativo em indivíduos obesos [89]. Do mesmo modo, um maior índice de massa corporal (IMC) entre os indivíduos com excesso de peso foi mostrado ser associado com aumento do risco de danos no DNA devido ao estresse oxidativo [43] e da doença cardiovascular [4, 37]. Resultados ainda não publicados do nosso grupo de pesquisa relacionados aos mesmos pacientes aqui investigados, mostraram que os indivíduos do grupo 2 apresentaram níveis elevados de peroxidação lipídica e de citocinas inflamatórias (IL-1α, IL-6, IL-8, TNF-α) no plasma. Nossa hipótese é que estes resultados não publicados juntamente com os altos níveis de Proteína C-reativa, triglicerídeos e IMC encontrados aqui para os pacientes do grupo 2, provavelmente levariam a excesso de ROS, causando efeitos celulares de danos no DNA. Portanto, mesmo que os indivíduos do grupo 2 tenham alcançado o controle glicêmico, verificou-se aqui que os danos ao DNA permaneceram em suas células do sangue periférico.

Considerando a avaliação periodontal realizada, sabe-se que a destruição periodontal pode ser causada ou aumentada pelas ROS e pelas proteases ativas que são lançadas durante a resposta imuno-inflamatória do hospedeiro frente ao desafio bacteriano [6, 15, 96, 114]. No entanto, há pouca informação considerando o papel da

produção de ROS por PMN ativos que podem levar ao dano oxidativo das moléculas de DNA [39, 104]. No presente estudo, encontramos índices estatisticamente maiores de MN, como FCM, FMN e presença de pontes nucleoplasmásticas no Grupo 4 (pacientes com periodontite crônica), em relação ao Grupo 5 (pacientes totalmente saudáveis). Este é o primeiro estudo a demonstrar associação da ocorrência de danos irreversíveis ao DNA com a periodontite crônica. Apesar do fato da periodontite ser uma doença inflamatória local e os índices de MN ter sido avaliado sistemicamente, nosso achado indica que a condição inflamatória parece ter ultrapassado os limites locais do periodonto e influenciou danos ao DNA de modo sistêmico. Entretanto, mais estudos são necessários para investigar em uma população maior, ou in vitro, danos ao DNA e/ou estresse oxidativo relacionado com a doença periodontal.

Neste estudo nós investigamos a hipótese que a ocorrência do DM2, dislipidemia e periodontite, que estão envolvidas nos processos de estresse oxidativo, podem aumentar proporcionalmente os danos ao DNA. Nossos resultados demonstraram que estas três patologias ocorrendo simultaneamente aumentaram as lesões ao DNA. Esse resultado pode ter sido causado pelo quadro inflamatório juntamente com o aumento do estresse oxidativo. Além disso, também foi possível demonstrar que o teste do micronúcleo foi útil como biomarcador para danos ao DNA em indivíduos com doenças crônico-degenerativas.

Em relação à avaliação da expressão gênica pela técnica de *microarray*, este é o primeiro estudo da literatura que investigou o perfil de expressão genética em indivíduos afetados simultaneamente por DM2, dislipidemia e periodontite crônica. Quando avaliamos o perfil de expressão gênica baseado no genoma dos (Grupo 1 + Grupo 2) *versus* Grupo 3, buscou-se saber quais genes seriam diferencialmente expressos sob a influência do DM2. Os resultados do *microarray* foram validados por

meio de RT-qPCR e demonstraram que o gene *HLA-DQA1* está hiper-expresso em diabéticos com dislipidemia e periodontite (Grupo 1 + 2) em comparação com o Grupo 3. O gene *HLA-DQA1* (*(Major Histocompatibility Complex, class II, DQ Alpha-1,* OMIM 146880*)* pertence às moléculas de HLA de classe II, que têm sido implicados na patogênese de várias doenças auto-imunes, devido ao seu papel central na apresentação de peptídeos antigênicos de células T auxiliares. A associação dos genes *HLA* de classe II na patogênese do diabetes mellitus tipo 1 tem sido relatada em várias etnias [59, 69, 108]. No entanto, estudos que investigaram a associação de HLA de classe II com diabetes tipo 2 têm revelado resultados inconsistentes, uma vez que foram relatadas associação positiva [73], nenhuma associação [14], e associação fraca entre HLA classe II e diabetes mellitus tipo 2. Em dois estudos, utilizando diferentes métodos de genotipagem, não foi encontrada associação entre o diabetes tipo 2 e o gene *HLA* de classe II (*HLA-DR, HLA-DQ*) em Punjabi Sikhs [42], enquanto uma associação positiva com os genes *HLA-DQA* foi relatado para indivíduos belgas [35]. No Bahrain, numa população com alta prevalência de diabetes tipo 2, esta doença foi associada significativamente com ambos os genótipos *HLA-DRB1* e *HLA-DQB1*, com alguns alelos conferindo suscetibilidade e outros desempenhando um papel protetor [73]. Ao que nos consta, não há qualquer estudo na literatura enfocando a expressão do gene *HLA-DQA1* com o diabetes tipo 2, dislipidemia e periodontite crônica ocorrendo simultaneamente nos mesmos pacientes. Mais estudos precisam ser realizados para elucidar os mecanismos que podem estar envolvidos na expressão do gene *HLA-DQA1* com a fisiopatologia das doenças aqui investigadas.

Outro gene que foi validado pelos resultados do *microarray* foi o gene *PDCD6*, que foi encontrado hipo-expresso no Grupo 3 em relação aos Grupos 1 + 2. O gene *PDCD6 (Programmed Cell Death 6,* OMIM 601057) está relacionado com a

desregulação do processo de apoptose, contribuindo para a patogênese de diversas doenças, incluindo doenças neurodegenerativas, câncer, doenças auto-imunes, malformações congênitas e imunodeficiência. O gene *PDCD6* codifica uma proteína de ligação do cálcio que pertence à família de proteínas penta-EF-hand e é expressa em todo o organismo [5, 68]. O gene *PDCD6* está hiper-expresso em tecido tumoral de pulmão, indicando que este pode desempenhar um papel na patologia de células cancerosas e/ou pode ser um marcador tumoral [58]. Por outro lado, a hipo-expressão do gene *PDCD6* foi associada com o desenvolvimento do câncer de ovário [47]. Interessantemente, no presente estudo foi encontrada pela primeira vez uma diminuição na expressão *PDCD6* em indivíduos com DM2 em comparação com normoglicêmicos, no contexto de dislipidemia e periodontite. Mais estudos são necessários para entender as razões da menor expressão do gene *PDCD6* nesses pacientes.

Em relação à comparação realizada entre os Grupo 1 *versus* Grupo 2, foi encontrado como resultado a hiper-expressão do gene *TRDV3* no Grupo 1. A literatura mostra pouca informação sobre este gene. O possível envolvimento do gene *TRDV3* (*t-cell receptor delta chain variable gene cluster*; OMIM 615459) em processos imuno-inflamatórios não é compreendido e não foi encontrado nenhum estudo investigando a expressão desse gene em qualquer uma das três doenças aqui investigadas, especialmente tendo em vista o controle glicêmico do diabetes tipo 2. Os genes da subfamília *TRDV* foram analisados em células PBMC de pacientes com a forma idiopática crônica (imunológica) de púrpura trombocitopênica (ITP) [125]. A ITP é uma doença auto-imune em que os anticorpos anti-plaquetários induzem a destruição das plaquetas, devido a um desequilíbrio da resposta imune. Os genes *TRDV1* e *TRDV2* puderam ser detectados na maior parte das amostras de ITP, bem como em controles saudáveis, e o gene *TRDV3* pode ser detectado em apenas dois casos de 11 casos com

ITP, e puderam ser encontrados em 90% dos controles saudáveis (p = 0,02). Em conclusão, a alteração do padrão periférico de *TRDV* pode desempenhar um papel na patogênese da destruição plaquetária imunomediada, em alguns casos com ITP.

O gene *PPAP2B (Phosphatidic acid phosphatase type 2B,* OMIM 607125*)* foi validado no presente estudo como hiper-expresso em diabéticos compensados metabolicamente com dislipidemia e periodontite (Grupo 2) em relação ao Grupo 1. O gene *PPAP2B* pertence à família das fosfatases de fosfato lipídico, que é composta por três membros. O gene *PPAP2B* codifica uma glicoproteína de membrana que contém seis domínios transmembrana amplamente expressa no organismo, que hidrolisa fosfatos lipídicos incluindo ácido lisofosfatídico (LPA) e esfingosina 1-fosfato. A hiper-expressão do gene *PPAP2B* demonstrou diminuir o crescimento, sobrevivência e tumorigênese de células de câncer de ovário [106]. Bianchini et al. [9] (2013) revelou que a diferenciação em adipócitos a partir de células mesenquimais humanas obtidas de tecido adiposo, foi associada com uma diminuição significativa da expressão do gene *PPAP2B*, sugerindo que este gene pode desempenhar um papel na adipogênese.

O gene *HLA-DQB1* (*Major Histocompatibilty Complex, class II, DQ Beta-1,* OMIM 604305*)* foi validado neste estudo como hiper-expresso em indivíduos do grupo 1 (diabéticos descompensados metabolicamente) em relação ao grupo 3 (normoglicêmicos). A suscetibilidade a algumas doenças infecciosas tem uma associação genética com alelos de MHC [82]. Ohyama et al. [82] (1996) e Takashiba et al. [105] (1999) relataram que o genótipo de *HLA-DQB1* está associado com início de periodontite agressiva. Além disso, o gene *HLA-DQB1* foi associado com a resposta auto-imune contra as células produtoras de insulina, levando ao desenvolvimento do diabetes tipo 1. Forbes et al. [27] (1995) não encontrou relação direta entre a suscetibilidade genética definida por *DQB1* e anticorpos contra células das ilhotas

pancreáticas em pacientes com diabetes tipo 1, o que sugere que as variações no locus *DQB1* não estão ligadas à expressão deste marcador auto-imune da destruição das células beta. Os mecanismos pelos quais as moléculas de *HLA-DQ* conferem suscetibilidade ao diabetes tipo 1 não são completamente compreendidos, no entanto, a influência dos desafios ambientais e imunológicos, em um indivíduo geneticamente predispostos foi reconhecida [75].

Também comparando os resultados de *microarray* do Grupo 1 *versus* Grupo 3, os genes *RIN3* e *VCAN* foram validados como hipo-expressos em indivíduos normoglicêmicos (grupo 3) em comparação com os diabéticos descompensados metabolicamente (Grupo 1). O gene *RIN3 (Ras* and *Rab* interactor 3, OMIM 610223*)* exibe um padrão de expressão tecido-específica, com níveis mais elevados nos mastócitos humanos. Kajiho et al. [50] (2003) sugere que *RIN3*, *Rab5*, e amfifisina II formam um complexo ternário que está envolvido na via de transporte endocítica precoce. Chung et al. [16] (2012) mostrou que polimorfismos genéticos no gene *RIN3* resultam num risco aumentado de desenvolver a doença de Paget. Costa et al. [19] (2007) revelou um grupo de genes associados à regulação da transcrição e tradução, entre eles o gene *RIN3*, sugerindo um possível papel na melhora dos sintomas clínicos de pacientes com anemia falciforme. Na literatura, não foi encontrada nenhuma informação da expressão deste gene com DM2, dislipidemia ou periodontite crônica. Encontramos também que o gene *VCAN* está hiper-expresso em diabéticos descompensados metabolicamente (Grupo 1) em comparação com indivíduos normoglicêmicos (Grupo 3). O gene *VCAN* (*Versican*) (também conhecido como *CSPG2*) codifica um proteoglicano sulfato de condroitina, que é um dos principais componentes da matriz extracelular (ECM), expresso em quase todos os tecidos. Enquanto *Versican* desempenha um papel no desenvolvimento de tecido normal, níveis elevados deste têm

sido relatados na maioria dos tumores malignos, incluindo os tumores não sólidos, tais como a leucemia monocítica aguda humana [67, 77, 90]. Novas evidências indicam que o aumento da expressão de *VCAN* está fortemente associado a resultados ruins para muitos tipos diferentes de câncer [40, 56, 86-88, 90, 91].

Considerando a comparação entre o Grupo 2 *versus* Grupo 3, a análise do *microarray* e RT-qPCR demostraram que o gene *PPIC* está hiper-expresso em diabéticos compensados metabolicamente com dislipidemia e periodontite (grupo 2) em comparação com o grupo 3. O gene *PPIC (Peptidyl-Prolyl Isomerase C,* OMIM 123842*)*, também conhecido como Ciclofilina C, codifica uma proteína de ligação celular para o imunossupressor Ciclosporina A, que pode suprimir a ativação de células T [29, 51, 62]. Matsuo et al. [71] (2013) avaliando a expressão gênica do carcinoma gástrico avançado usando miRNA *microarray*, descobriu que os genes *RCC2* e *PPIC* estavam hiper-expressos nesses tecidos e, portanto, ambos foram identificados como possíveis alvos de miR-29c no carcinoma gástrico. Além disso, Obermayer et al. [81] (2013) identificaram uma superexpressão do gene *PPIC*, avaliando os novos marcadores de células tumorais circulantes (CTCs) em pacientes com câncer epitelial de ovário. Estes fatos levaram-nos a especular que a superexpressão do gene *PPIC* pode afetar alguns aspectos da resposta imune, como a inflamação durante a ocorrência do DM2. No entanto, para avaliar a função do gene *PPIC* no contexto da fisiopatologia do diabetes, são necessários mais estudos. Nenhuma informação foi encontrada sobre a expressão desse gene com as doenças aqui estudadas. Finalmente, o gene *SLC6A13* foi validado neste estudo, sendo encontrado hipo-expresso nos pacientes do grupo 2 em relação ao grupo 3 (normoglicêmicos). O gene *SLC6A13 (Solute carrier family 6- neurotransmitter transporter, gaba-member 13,* OMIM 615097), também conhecido como Transportador GABA tipo 2 (*GAT2)*, codifica o transportador para o ácido gama-aminobutírico

(GABA) e taurina em tecidos periféricos e parece funcionar na manutenção dos níveis no plasma destes solutos [126]. O genoma dos mamíferos contém quatro genes que codificam os transportadores de GABA de alta afinidade, e o gene *SLC6A13* é um deles [38, 63]. A maior parte do interesse nestes transportadores é em relação ao seu papel na sinalização de inativação de GABA no sistema nervoso central [18, 23, 30]. Nem a função exata do gene *SLC6A13* nem seus níveis de expressão foram determinados na literatura, mas sabe-se que este gene é altamente conservado entre as espécies. GABA pode desempenhar um papel na sinalização de órgãos periférico, incluindo o sistema imune [33, 123]. No entanto, a contribuição potencial do gene *SLC6A13* em processos imuno-inflamatório não é compreendido, e na literatura não encontramos nenhum estudo investigando a expressão desse gene no diabetes tipo 2, nem vinculado com dislipidemia e periodontite.

Finalmente, o presente estudo explorou em 54,675 transcritos humanos aqueles que poderiam ser expressos de maneira diferente em cada condição patológica aqui investigada. A partir dos genes diferencialmente expressos identificados, mais estudos são necessários para compreender as razões biológicas para a associação com a condição metabólica específica. Concluímos que identificamos genes diferencialmente expressos em células mononucleares do sangue periférico de pacientes com diabetes tipo 2 compensados e descompensados metabolicamente, também afetados pela dislipidemia e periodontite crônica. Esses genes são candidatos para futuras investigações para avaliar se eles poderiam ser considerados marcadores em sangue periférico das patologias aqui estudadas. Além disso, no futuro, estes genes poderiam ser avaliados como possíveis novos alvos terapêuticos que podem regular de forma seletiva as doenças aqui investigadas.

5 CONCLUSÃO

Dentro das limitações deste estudo, os resultados obtidos permitem concluir que:

1. Foi encontrada maior frequência de micronúcleos em pacientes com diabetes tipo 2 compensados metabolicamente apresentando dislipidemia e periodontite crônica, indicando que a ocorrência simultânea dessas três patologias promovem um papel adicional para produzir lesões ao DNA.

2. O teste do micronúcleo foi útil como biomarcador para danos ao DNA em indivíduos com doenças crônico-degenerativas.

3. Foram identificados e validados genes diferencialmente expressos em células mononucleares do sangue periférico de pacientes com diabetes tipo 2 compensados/descompensados metabolicamente com dislipidemia e periodontite crônica.

- Foram validados os seguintes genes: *HLA-DQA1*, *PDCD6, TRDV3, PPAP2B, HLA-DQB1, RIN3, VCAN, PPIC* e *SLC6A13.*

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7 APÊNDICE

7.1 Apêndice 1 – Material e Método

Descrição da amostra

Os pacientes foram selecionados dentre os que buscaram atendimento na Faculdade de Odontologia de Araraquara e os que foram encaminhados pelas Unidades Básicas de Saúde do município e região. Após esclarecimento da natureza e objetivos da pesquisa, os pacientes confirmaram sua aceitação para participar do estudo mediante a assinatura de um Termo de Consentimento Livre e Esclarecido, previamente aprovado pelo Comitê de Ética em Pesquisa em Seres Humanos (Protocolo nº 50/06) (Anexo A). Este estudo foi conduzido entre Maio de 2009 e Junho 2011.

Os participantes desta pesquisa receberam orientação sobre a doença periodontal e, após as coletas de material, foram submetidos a tratamento periodontal. Os pacientes portadores de diabetes, quando necessário, foram encaminhados ao endocrinologista responsável para acompanhamento e eventual tratamento da condição metabólica.

Grupos

O cálculo da amostra foi baseado em um estudo piloto conduzido pelo nosso grupo de pesquisa, em que observando uma diferença de 3.0 unidades na concentração de um determinado marcador de peroxidação lipídica (MDA) e usando um desvio padrão de 1.4 unidades, poderia ser observada uma diferença significativa entre o um grupo de pacientes com diabetes quando comparado a um grupo sem diabetes. Desta forma, foi calculado que seriam necessários pelo menos 20 pacientes em cada grupo para termos um poder de 90% com um intervalo de confiança de 95%. Tendo em vista a possibilidade de desistência de

pacientes em participar do estudo durante as fases de execução, cada grupo foi constituído de 30 pacientes.

Assim, a amostra foi composta por 150 indivíduos, divididos em cinco grupos:

- GRUPO 1 (n=30) – Indivíduos portadores de diabetes mellitus tipo 2, descompensados metabolicamente (HbA1c ≥ 8,0%), com dislipidemia [74] e com periodontite crônica [49].
- GRUPO 2 (n=30) – Indivíduos portadores de diabetes mellitus tipo 2, compensados metabolicamente (HbA1c < 8,0%), com dislipidemia [74] e com periodontite crônica [49].
- GRUPO 3 (n=30) – Indivíduos sem diabetes mellitus tipo 2, com dislipidemia [74] e com periodontite crônica [49].
- GRUPO 4 (n=30) – Indivíduos sem diabetes mellitus tipo 2, sem dislipidemia [74] e com periodontite crônica [49].
- GRUPO 5 (n=30) – Indivíduos sem diabetes mellitus tipo 2, sem dislipidemia [74] e sem periodontite crônica [49].

Para o grupo de indivíduos portadores de diabetes, foi realizado exame laboratorial para avaliação da HbA_{1c} e, a partir deste resultado, o paciente foi alocado no grupo 1 (compensado metabolicamente) ou no grupo 2 (descompensado metabolicamente). Para os indivíduos dos demais grupos (Grupos 3, 4 e 5), o exame de glicemia de jejum foi realizado para confirmação da ausência do diabetes, sendo considerados normoglicêmicos aqueles com resultado inferior a 100mg/dl.

O critério de $HbA_{1c} \geq 8,0\%$, no grupo de pacientes metabolicamente descompensados, foi escolhido para evitar a inclusão de pacientes com descompensação metabólica transitória e foi baseado em estudos prospectivos e randomizados [1] que

definem o limite de 7% da HbA_{1c} como controle meatabólico aceitável. Assim, a inclusão de pacientes com 1% acima do limite superior eliminará a interferência de possíveis alterações metabólicas transitórias.

Critérios de Inclusão

- Ambos os sexos
- Idade de 35 a 60 anos
- Presença de, no mínimo, 15 dentes
- Apresentar pelo menos 4 sítios com PS ≥ 5mm, NI ≥ 4mm e sangramento à sondagem, em dentes não-adjacentes.

Critérios de Exclusão

- Antibioticoterapia nos seis meses e de antiinflamatórios esteróides ou não-esteróides nos três meses antecessores ao estudo
- Gestação e lactação
- Uso de anticoncepcional ou qualquer outra forma de hormônio
- Tabagismo
- Presença de anemia
- Tratamento periodontal nos últimos 6 meses
- Uso de drogas hipolipemiantes: vastatinas, estatinas, inibidores da HMG-CoA redutase, resinas de troca, fibratos, ácido nicotínico, ômega-3, ácido acetilsalicílico (AAS), inibidores da enzima de conversão (IECA), betabloqueadores (BB)

- Uso de drogas que interferem no metabolismo lipídico: anti-hipertensivos, imunossupressores, esteróides, anticonvulsivantes, ácido acetilsalicílico, ácido ascórbico, amiodarona, alopurinol, glitazonas
- História de doenças que interferem no metabolismo lipídico: hipotireoidismo, hipopituiritarismo, síndrome nefrótica, insuficiência renal crônica, atresia biliar congênita, doenças de armazenamento, lúpus eritematoso sistêmico.

Procedimentos Clínicos

Anamnese

Todos os pacientes foram submetidos a um questionário estruturado com relação aos dados demográficos, história de saúde geral e bucal. Os pacientes portadores de diabetes foram avaliados quanto à história pregressa da doença, presença de complicações associadas ao diabetes, medicações utilizadas para controle metabólico, dentre outras informações por meio de uma ficha elaborada especialmente para a Clínica de Pacientes Portadores de Diabetes da Faculdade de Odontologia de Araraquara.

Exame Físico

Os pacientes foram submetidos a um exame físico no qual foram avaliados: circunferência abdominal, proporção cintura/quadril e peso e altura para obtenção do índice de massa corporal (IMC) de cada indivíduo.

Exame Clínico Intrabucal e Radiográfico

Todos os pacientes foram submetidos ao exame periodontal completo, realizado por um único examinador treinado e previamente calibrado (kappa=0,89), usando instrumental da marca Hufriedy®

A avaliação periodontal foi composta de índice de placa visível (IPV), índice de sangramento marginal (ISM), sangramento à sondagem (SS), mensuração da profundidade de sondagem, posição da margem gengival e nível clínico de inserção (NI). Foi realizado exame radiográfico de boca toda (nos pacientes dos Grupos 1, 2, 3 e 4) para avaliação da perda óssea periodontal.

Foi considerado nesse estudo:

- Índice de placa visível: presença ou não de placa bacteriana visível a olho nu, após secagem da superfície dentária com jato de ar, em todas as faces de todos os dentes;
- Índice de sangramento marginal: presença ou ausência de sangramento marginal após percorrer o espaço do sulco de uma proximal a outra, com a sonda periodontal milimetrada inclinada em 60 graus em relação ao dente;
- Posição da margem gengival: distância da margem gengival à junção cemento-esmalte;
- Mensuração da profundidade de sondagem: distância da margem gengival ao fundo do sulco gengival, medida com sonda periodontal milimetrada em 6 sítios por dente: disto-vestibular, vestibular, mesio-vestibular, disto-lingual, lingual e disto-lingual;
- Sangramento à sondagem: presença ou ausência de sangramento, decorrido um tempo de 30 segundos depois de mensurada a profundidade de sondagem;

- Avaliação do nível clínico de inserção: corresponde à somatória das medidas da posição da margem gengival e profundidade de sondagem, para cada sítio de cada elemento dentário.

Procedimentos radiográficos: todos os pacientes dos Grupos 1, 2, 3 e 4 foram submetidos a exame radiográfico de boca toda (radiografias periapicais) realizadas com auxílio de posicionadores autoclaváveis da marca RINN; filme periapical kodak e um aparelho de raio-X odontológico convencional. Posteriormente, os filmes radiográficos serão processados na processadora automática Dent – X 9000 utilizando soluções químicas kodak e montados em cartelas apropriadas para interpretação das imagens em negatoscópio.

Uma classificação de periodontite crônica severa foi definida para aqueles pacientes que apresentavam profundidade de sondagem (PS) ≥ 6mm, nível de inserção (NI) ≥ 5mm e sangramento à sondagem em pelo menos 8 sítios distribuídos em diferentes quadrantes [57].

Procedimentos Laboratoriais

Análise laboratorial do controle metabólico e perfil lipídico

Após um jejum de 12 horas, os pacientes foram encaminhados a um Laboratório de Análises Clínicas para coleta do sangue por punção venosa. Foi utilizado o equipamento COBAS MIRA plus para realização dos exames: Hemograma (ABX Micros 60), Insulina (método quimioluminescência), Glicemia de Jejum (método Bondar e Mead modificado) (Kit Labtest), HbA_{1c} (método de imunoensaio de inibição turbidimétrica) (Kit Roche), Proteína C-reativa ultrasensível (método nefelometria),

Triglicérides (método enzimático-Trinder) (Kit Labtest), Colesterol Total (método enzimático-Trinder) (Kit Labtest) e HDL-colesterol (método enzimático) (Kit Labtest).

As frações do VLDL e LDL-colesterol foram calculadas segundo a equação de Friedewald (válida se Triglicérides <400mg/dl) [101]:

Colesterol VLDL = triglicérides / 5

Colesterol LDL= Colesterol Total – (HDL+VLDL)

Para evitar a inclusão de pacientes com dislipidemia transitória, para os grupos 1, 2 e 3, foram considerados os limites superiores de cada fração lipídica, de acordo com o National Cholesterol Educational Program (NCEP) Adult Treatment III (ATP III) [74]:

- **Colesterol Total (CT):** Considerado alto para valores ≥ 240 mg/dl.
- **LDL (Low density lipoprotein):** Considerado alto para valores ≥ 160 mg/dl.
- **HDL (High density lipoprotein):** Considerado alto para valores ≥ 40 mg/dl.
- **Triglicérides (TG):** Considerado alto para valores ≥ 200 mg/dl.

Coleta das Amostras de Plasma Sanguíneo

Foi coletado de cada paciente no mesmo Laboratório de Análises Clínicas, por meio de punção venosa, sangue em tubo Vacutainer contendo EDTA para posterior análise da expressão gênica por meio da técnica do m*icroarray* e sangue em tubo Vacutainer contendo Heparina para posterior análise de danos no DNA (teste do Micronúcleo).

Teste de Mutagenicidade - Micronúcleo

O teste do micronúcleo (MN) foi utilizado para avaliar as freqüências de MN presentes em linfócitos do sangue periférico dos pacientes dos diferentes grupos. Por meio desse teste será avaliado se a descompensação do Diabetes tem efeito mutagênico, levando a quebras irreparáveis nos cromossomos dos pacientes. Foi adotada a metodologia proposta por Fenech, Morley [26] (1985).

De todos os pacientes selecionados (dos 5 grupos experimentais), os linfócitos obtidos a partir de 5mL de sangue coletado em tubo com heparina foram cultivados em duplicata em 5mL meio de cultura completo constituído de 78% de meio RPMI 1640 (Sigma), 20% de soro bovino fetal (Gibco) e 2% de fitohemaglutinina (Gibco), suplementado com antibióticos penicilina (0,005 mg/mL) e estreptomicina (0,01 mg/mL). O tempo total de cultivo foi de 72 h em estufa a 37ºC com atmosfera de CO_2 a 5% sendo que, após 44 h de cultivo, foi adicionado a cada cultura 6μg/mL de citocalasina B (Sigma) para a inibição da citocinese. As culturas retornaram à estufa por mais 28 h até ser completadas as 72 h de cultivo.

Para a colheita das células em cultivo, as culturas foram centrifugadas por 5 minutos e o sobrenadante foi descartado. O sedimento celular foi homogeneizado com 3mL de uma solução hipotônica de citrato de sódio (1%) gelada e imediatamente as células foram fixadas com 3mL de fixador metanol:ácido acético (3:1) e 4 gotas de formaldeído (37%). Esse material foi centrifugado, o sobrenadante descartado e o sedimento celular lavado mais duas vezes em 5mL de fixador metanol:ácido acético (3:1). Após a última lavagem o material foi centrifugado e ressuspenso em apenas 0,5mL de fixador. Essa suspensão celular foi gotejada sobre lâminas previamente limpas e mantidas em água destilada gelada.

A coloração do material foi realizada usando-se solução de Giemsa diluído em tampão Sörensen ($Na2HPO4$ e $KH2PO4$ a 0,06M, pH 6,8) na proporção 1:20 por 5 minutos, quando então as lâminas foram enxaguadas em água corrente, secas à temperatura ambiente e armazenadas para posterior análise.

A análise das lâminas foi realizada em microscópio óptico em aumento de 40 vezes. Foram contabilizadas por lâmina 1000 células binucleadas com citoplasma íntegro e núcleos principais nitidamente delimitados, sendo anotadas as células binucleadas que continham micronúcleos (1, 2 ou 3). Foram considerados como micronúcleos os fragmentos com tamanho entre 1/16 e 1/3 dos núcleos principais, com coloração semelhante à coloração dos núcleos principais, sem emissão de refringência e sem sobreposição a qualquer um dos núcleos principais. Para a obtenção do Índice de Divisão Nuclear (IDN) considerou-se a freqüência de células com 1, 2, 3 ou 4 núcleos em uma população de 1000 células analisadas. O IDN foi calculado pela seguinte fórmula:

$$IDN = \frac{M1 + 2(M2) + 3(M3) + 4(M4)}{1000}$$

Onde, M1 a M4 representam: número de células com 1, 2, 3 e 4 núcleos respectivamente e n=1000, representa o número total de células viáveis.

Separação de leucócitos para análise da expressão gênica

Os tubos Vacutainer contendo o sangue dos pacientes selecionados para os 5 grupos foram mantidos em gelo e os leucócitos do sangue foram separados imediatamente por meio de centrifugações com Ficoll Paque Plus (GE Healthcare) e

consecutivas lavagens com Salina (NaCl 0,9%). Uma vez que os *pellets* de leucócitos foram obtidos, foi realizada sua ressuspensão em TRIzol® (Invitrogen, Carlsbad, CA), e estes então foram armazenados a -80°C para posterior extração do RNA.

Análise da Expressão Gênica

Extração RNA

A extração do RNA total de todas as amostras foi realizada por meio do TRIzol® (Invitrogen, Carlsbad, CA) segundo o protocolo recomendado pelo fabricante. A purificação das amostras foi realizada pelo kit RNeasy Protection Mini Kit (Qiagen). A concentração do RNA extraído de cada amostra foi mensurada por densidade óptica por espectroscopia (NanoVue Spectrophotometer, GE Healthcare Life Sciences, Oslo, Noruega) e a qualidade do RNA foi checada por eletroforese em gel de agarose a 1%. Foram utilizadas amostras de RNA com razão 260/280 e 260/230 entre 1,8 a 2,2, e após diluição a 33,3 ng/ul, estas foram armazenadas a -80ºC até o momento de realizar os procedimentos do *microarray*.

Técnica do *Microarray* - Sistema Affymetrix

O RNA purificado e diluído foi transportado em gelo seco de Araraquara até a Faculdade de Ciências Médicas da UNICAMP onde foi realizada esta parte do trabalho. Foram utilizados os *chips* Human Genome U133 Plus 2.0 Array, que contém 54.675 transcritos de modo a cobrir o genoma. As sequências de cada *chip* são derivadas dos bancos de dados GenBank®, dbEST e RefSeq. Os dados completos, incluindo anotações das sequências e dos genes podem ser adquiridos no NETAFFX™ (www.affymetrix.com/analysis/index.affx). Também foram usados os kits 3'IVT Express e Hybridization Wash and Stain para a obtenção do cDNA (DNA

complementar), aRNA (RNA amplificado), fragmentação do aRNA, marcação, hibridização, detecção e lavagem. O protocolo completo teve duração de 3 dias e foram realizados 5 *chips* por vez. A lavagem e escaneamento dos *chips* foi realizada no Laboratório de Microarranjos do Laboratório Nacional de Luz Síncrotron (LNLS) por meio do GeneChip Scanner 3000 (Affymetrix).

O protocolo seguiu a recomendação do fabricante e está descrita resumidamente a seguir.

Preparação dos Alvos

Neste passo, uma dupla fita de cDNA foi sintetizada a partir do RNA total extraído das amostras. Para purificação da dupla fita de cDNA foi usado o kit GeneChip Sample Cleanup. A partir deste cDNA, foi realizado uma reação de transcrição *in vitro* para a produção de cRNA marcado com biotina usando-se o kit GeneChip IVT Labeling. Desta reação, foram utilizados 20µg de cRNA (RNA complementar) para fragmentação antes da hibridização.

Hibridização e Detecção

Foi preparado um *cocktail* contendo o alvo fragmentado e os controles de hibridização. Este *cocktail* foi colocado dentro do *chip* e incubado em forno de hibridização por 16 horas a 45°C em rotação de 60rpm.

Lavagem e Detecção

Imediatamente após a hibridização, os *chips* foram colocados na estação fluídica para serem automaticamente lavados e marcados. Para isso soluções de estreptoavidina,

e anticorpo biotinilado foram colocados na estação fluídica como indicado pelo manual para lavagem e detecção.

Aquisição das Imagens e Análise dos Dados

As imagens de cada *chip* foram adquiridas com o GeneChip Scanner 3000. O *software* define cada célula do *chip* e computa a intensidade para cada uma destas células. Cada *chip* teve sua imagem armazenada em separado e identificada pelo nome. Os arquivos brutos de leitura foram armazenados para a posterior análise. A quantificação da intensidade do sinal é feita pela intensidade média dos pixels (menor ponto que forma uma imagem digital). A imagem foi analisada por intensidade e os resultados foram visualizados em formatos gráficos e tabulares. Os cálculos para a primeira análise dos níveis de expressão e dos transcritos ausentes/presentes foram calculados com o pacote Affy [32] do Bioconductor [34] no ambiente R usando-se o algoritmo MAS5.0, com posterior análise estatística também utilizando o ambiente R. Também foram empregados *softwares* de vias de interações gênicas como o MetaCore^{TM} Pathyway Analysis (http://www.genego.com/metacore.php).

Validação dos resultados do Microarray por PCR em Tempo Real ou quantitativo (RT-qPCR)

Efetivou-se a avaliação por *microarray* em 6 pacientes por grupo e a validação desses resultados foi realizada em amostras de RNA do total de pacientes de cada grupo (n=24). Foi sintetizado o DNA complementar (cDNA) de todas as seqüências gênicas expressas por meio do RT-PCR utilizando o Oligo $dT_{(20)}$ com o kit *SuperScript III First-Strand Synthesis Super Mix* (Invitrogen), de acordo com as instruções do fabricante.

As reações de PCR em tempo real foram realizadas pelo sistema *TaqManTM* (Applied Biosystems, Foster City), que é constituído por um par de primers e uma sonda marcada com um fluoróforo. Os primers foram desenhados segundo os genes que deverão ser validados. A sonda escolhida foi marcada com o fluoróforo FAM. Tínhamos proposto utilizar dois genes para controle endógeno da reação, mas decidimos utilizar três genes para normalizar com confiabilidade a expressão do gene de interesse nas diferentes amostras. Foi realizada a validação do sistema gene de interesse /controle endógeno, a fim de verificar se as eficiências de amplificação de ambos os genes são semelhantes e próximas a 100%. Para a quantificação relativa do gene selecionado, as reações de PCR em tempo real foram realizadas em triplicata a partir de: 6,25μL de *TaqMan Universal PCR Master Mix* 2x, 0,625μL da solução de primers e sonda, 1,625μL de água e 4,0μL de cDNA (50ng), sendo que no controle negativo, foi adicionado 4,0 μL de água ao invés do cDNA. As condições de ciclagem utilizadas foram: 50°C por 2 minutos, 95°C por 10 minutos e 40 ciclos de 95°C por 15 segundos e 60°C por 1 minuto. Os valores da expressão gênica relativa foram obtidos pela análise dos resultados no programa *7500 System SDS Software* (Applied Biosystems, Foster City). Os valores da expressão gênica relativa dos genes de interesse foram analisados utilizando o *software* Expression Suite (Applied Biosystems, Foster City). O *software* utiliza o método comparativo Cτ (ΔΔCt) e possibilitou quantificar com precisão a expressão gênica relativa dos genes analisados. Após normalização pela expressão dos genes constitutivos, foram obtidos os valores de expressão para cada gene alvo (ΔCt). A média dos valores de ΔCt de cada gene de interesse (alvo) referente a cada comparação entre os grupos foram comparados entre si. Os dados obtidos foram tabulados e os resultados conseguidos foram organizados em gráficos de coluna.

Análise Estatística

Os dados foram tabulados no programa Excel® 2007 e foram submetidos à análise estatística descritiva e analítica pelo programa GraphPad Prism 5.0. A distribuição e normalidade dos dados foram avaliadas pelo teste de D'Agostino & Pearson. As características gerais de cada grupo foram descritas utilizando media e desvio padrão (DP). As diferenças entre os grupos para dados paramétricos foram avaliadas pelo teste ANOVA, seguido do pós-teste de Bonferroni e os dados não paramétricos foram avaliados pelo teste Mann-Whitney ou Kruskall-Wallis seguido do pós-teste de Dunn. Testes de correlação de Pearson foram utilizados para dados com distribuição normal e de Spearman para dados não-normais, para investigar a correlação entre as diferentes variáveis. O nível de significância estabelecido foi de 5%. A hipótese de nulidade foi rejeitada se $p \leq 0.05$.

Para análise dos dados de *microarray* contamos com a parceria de Bioinformatas da FCM da UNICAMP e foi realizado teste t-student para verificar s expressão diferencial com o valor de $p \leq 0,01$ e o *Fold Change* (FC) $\geq \pm 2$ (razão entre as médias da expressão do transcrito nos grupos).

8 ANEXO

8.1 Anexo A - Certificado do Comitê de Ética

UNIVERSIDADE ESTADUAL PAULISTA " JÚLIO DE MESQUITA FILHO"

FACULDADE DE ODONTOLOGIA DE ARARAQUARA

Comitê de Ética em Pesquisa

Certificado

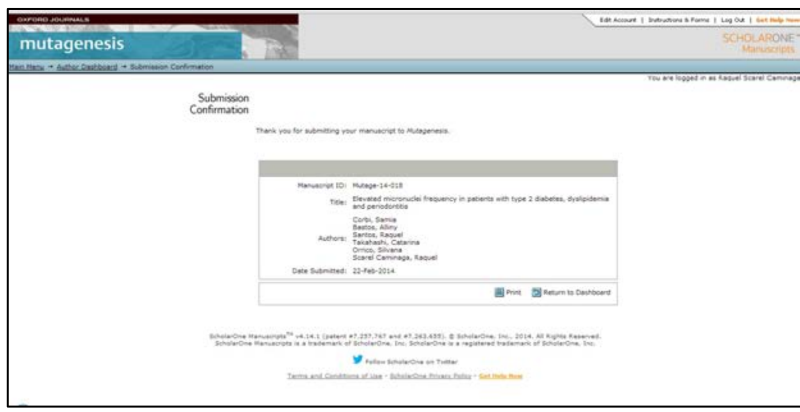
Certificamos que o projeto de pesquisa intitulado *"DIABETES MELLITUS E DOENÇA PERIODONTAL: ANÁLISE CLÍNICA, GENÉTICA, MICROBIOLÓGICA, IMUNOLÓGICA E BIOQUÍMICA"*, sob o protocolo nº **50/06**, de responsabilidade do Pesquisador (a) *SILVANA REGINA PEREZ ORRICO*, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde/MS, de 10/10/96, tendo sido aprovado pelo Comitê de Ética em Pesquisa–FOAr, com validade de 04 (quatro) anos, quando será avaliado o relatório final da pesquisa.

Certify that the research project titled *"DIABETES MELLITUS AND PERIODONTAL DISEASE: CLINICAL, GENETICAL, MICROBIOLOGICAL, IMMUNOLOGICAL AND BIOCHEMICAL ANALYSIS"*, protocol number **50/06**, under Dr *SILVANA REGINA PEREZ ORRICO*, responsability, is under the terms of Conselho Nacional de Saúde/MS resolution # 196/96, published on May 10, 1996. This research has been approved by Research Ethic Committee, FOAr-UNESP. Approval is granted for 04 (four) years when the final review of this study will occur.

Araraquara, 23 de abril de 2007.

Profª Drª *Mirian Aparecida Onofre*
Coordenadora

8.2 Anexo B - Documentos Comprobatórios



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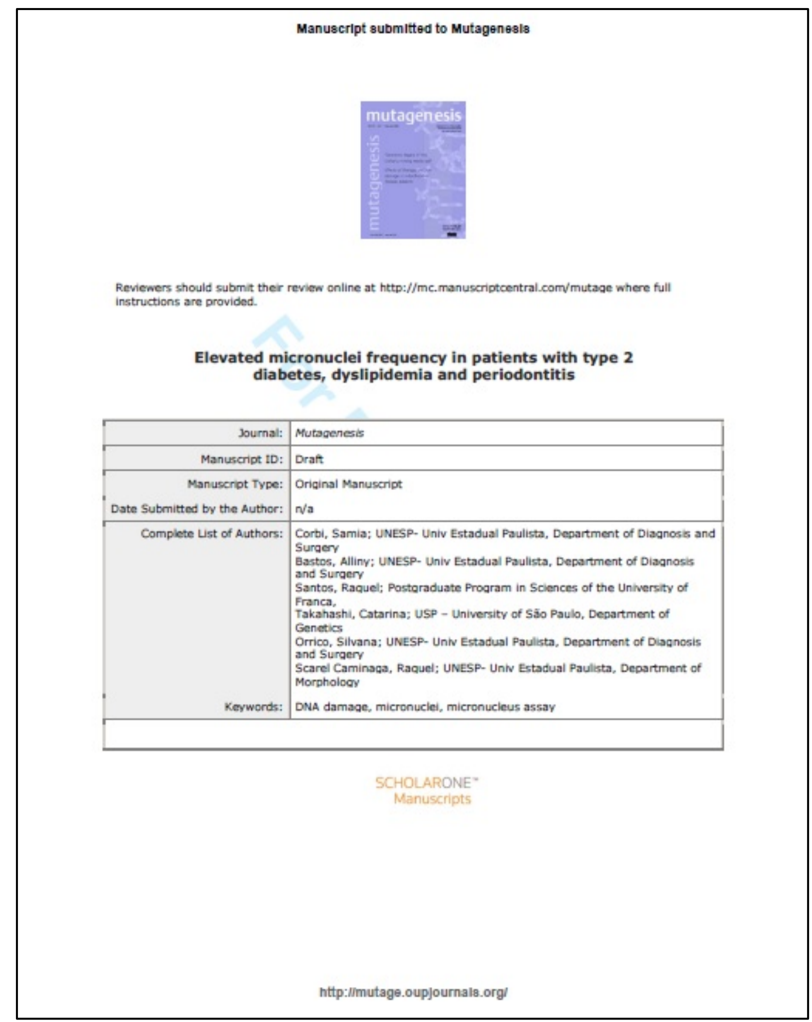

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SÂMIA CRUZ TFAILE CORBI